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Vol. XXXII. *April, 1917.* *No. 4*

BIOLOGICAL BULLETIN

EXPERIMENTAL ALTERATION OF THE AXIAL GRADIENT IN THE ALGA, GRIFFITHSIA BORNETIANA.

C. M. CHILD.

(WITH 15 FIGURES.)

The existence of axial gradients in susceptibility to various agents and conditions has been demonstrated as a characteristic feature of axiate animals, and the relation of such gradients on the one hand to metabolic and protoplasmic condition and on the other to the morphological and physiological order or pattern characteristic of development has been discussed in various publications.[1] The results of these investigations on animals led to the attempt to determine whether similar axial gradients occur in plants and some of the data on algæ have already appeared (Child, '16*c*, '16*e*, '17). The existence of such gradients in the axes of at least certain plants as well as in the axes of animals being demonstrated, it remains to determine their relation to the order or pattern of the plant axis. To accomplish this completely it would be necessary to obliterate or reverse existing gradients, to determine experimentally the origin of new gradients and to observe the effect of such changes upon order or pattern in the plant body.

Obliteration and reversal of axial gradients in algæ by various means have already been described (Child, '16*e*, '17). In most cases these changes represented a stage in the process of dying, and while they showed that changes might occur, the fact that these changes were soon followed by death made it impossible

[1] See Child, '15*a*, Chap. IX., '15*b*, '15*c*, '16*a*, '16*b*, '16*c*, '16*d*. Hyman '16, and references to further literature in these publications.

to determine their effect upon the developmental and morphological order in the plant body. In the present paper some experimental alterations of the gradient which do not necessarily end in death are described, and some evidence concerning the effect of these changes on further development and axiation is presented. This is merely the first step toward the experimental determination of the relation between the axial gradient and polarity in the plant and the very simple experiments described here serve chiefly to point the way to more exact determination and control.

For experimental purposes a form with large cells and sensitive enough to react readily to environmental changes but not too sensitive was desirable. I am indebted to Professor Osterhout for calling my attention to *Griffithsia*, which has proved to be of interest in various ways.

The Griffithsia Axis and Gradient.

The cell outline of an average, well-developed vegetative axis and branches from the material employed is indicated in Fig. 1, the hairs (Child, '17) being omitted. The method of growth is evident from the figure. The apical cell grows to a certain variable size and divides into a very small apical cell which repeats the process of growth and division, and a larger subapical cell, which continues to grow, but does not divide unless it later gives rise to a new axis by the separation of a new apical cell from some point of its apical region, as in the formation of new branches (Fig. 1). At a greater or less distance from the apical end a secondary basal elongation of the cells begins and is evident in the progressive change of shape of the cells toward the basal end of the axis (Fig. 1).

As already noted (Child '16*e*), the general susceptibility gradient in such an axis and in each branch is basipetal when the plant is in good condition. In the apical third or half of the length the gradient is very uniform, but in the basal region irregularities occur consisting in a susceptibility in one or more cells above or below the expectation corresponding to the level of the cell or cells. In the two or three most apical cells the intracellular gradient is usually slight or sometimes not visible,

but is basipetal when present. Often the apical cell itself shows a distinct basipetal gradient. Several cells from the third or fourth on usually show a distinct basipetal intracellular gradient, but with the beginning of the secondary basal elongation of the cell a basal region of high susceptibility usually appears, and some of these elongating cells show a double gradient, a region near the middle being the least susceptible. In these elongated cells of the more basal levels of well-developed axes the intra- cellular gradients may differ from cell to cell and from time to time, even though the general axial gradient remains essentially unaltered.

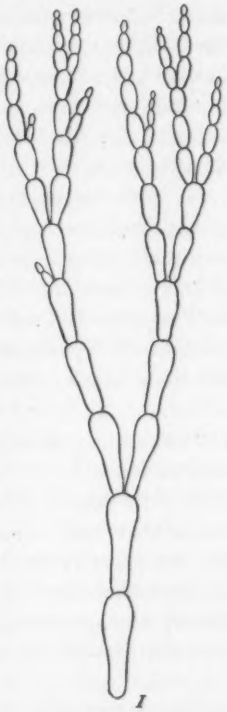


FIG. 1.

Not infrequently rhizoids develop on these more basal elongated cells even at a distance of several cells from the basal end of the axis, and in such cases the rhizoid may appear at any level, even close to the apical end of the cell, while the latter is still, at least physically, a part of the axis. In cases where such well-developed rhizoids are found on such cells it is of course impossible to determine the relation between the intracellular gradient and the point of origin of the rhizoid at the time when the rhizoid arose, and the possibility always exists that the intra-cellular gradient may have undergone alteration since the origin of rhizoid. Rhizoid development is primarily a feature of the basal end or the more basal regions of the axes of algæ (Tobler, '06), and as such we might expect to find it associated with regions of low susceptibility, *i. e.*, of low metabolic rate. The alterations in the intracellular gradients of the older more basal cells of *Griffithsia* suggest the possibility that even where a rhizoid arises from the middle or the apical region of such a cell this region may be the region of lowest metabolic rate in that particular cell.

The appearance of rhizoids or rhizoid-like structures on the older, more basal parts of the axes, is a characteristic feature of various species of algæ and in some cases these structures are so numerous as to form a more or less continuous cortical layer on such parts. In *Dasya*, for example, where such a secondary cortex formed by rhizoids is present on the older portions of the axes, the cells of this region show a relatively low susceptibility. In short, the rhizoid appears to be a structure originating at the lower levels of an axial metabolic gradient, though of course external conditions may determine whether or not rhizoids shall develop at levels where their development is possible.

Some of the experimental evidence presented below, as well as the alteration and reversal of the primary intracellular gradient in the more basal cells indicates very clearly that the degree of axiate individuation, *i. e.*, the permanence of the primary metabolic axial relations and their effectiveness in maintaining a particular order is not very high. Moreover, the short length which the axes attain, the slight connection between the cells, the irregularity of branching and the above-mentioned development of rhizoids from any level of the more basal cells all support this conclusion.

If physiological polarity is primarily a metabolic gradient in protoplasm, such a form as *Griffithsia* should be favorable material for the experimental obliteration of old axes or gradients and the establishment of new ones. The experiments described below, which are suggestive rather than conclusive, constitute a first step in this direction.

Experimental Obliteration and Reversal of the Gradient in Relation to Individuation.

The fact that obliteration or reversal of the general axial gradient of *Griffithsia* usually occurs during the first few days in the laboratory, even where the plants are kept in running water, has been noted in an earlier paper (Child, '16*e*). The results of a few simple experiments along this line remain to be considered.

Confinement in a limited supply of water without change in a closed flask without air was used as an extreme degree of "bad"

environmental conditions. In these experiments masses consisting of many axes were placed in 100 c.c. Erlenmeyer flasks which were filled with water, corked without air space and kept in the running water of the laboratory aquarium under the same conditions of light and temperature as the stock.

Susceptibility tests were made on the fresh material before confinement, and at intervals during the experiment the susceptibility to KCN *m*/100 or *m*/50 of portions removed from the flask was compared with that of controls from the same plants kept in running water, the phycoerythrin serving as indicator of death, as described in an earlier paper (Child, '16*e*).

The results of these experiments are briefly as follows: the plants of course die in the flask in a few days at most, in my experiments in 2–7 days, the survival time varying in general inversely as the amount of plant tissue present. The death gradient is basipetal as with other agents, *i. e.*, the apical regions are most susceptible to the confinement and the susceptibility decreases basipetally. The progress of death can be observed with the naked eye without difficulty, for each cell as it dies becomes orange-yellow by reflected light.

After twenty-four hours' confinement and before the death of any cells the susceptibility to KCN has decreased and the general susceptibility gradient in KCN shows a more or less complete reversal in the apical regions of most axes. This reversal may involve only the apical cell itself, in which case it dies later instead of earlier than the cell next below it, or it may include two, three or even the five or six most apical cells of the axis. In such cases these cells are less susceptible and die later than the cells next below, and in this group the death gradient from cell to cell is more or less distinctly acropetal, the apical cell dying last. Where the apical cell has recently divided and is very small it may still show a high susceptibility and the reversal may occur in the subapical region.

After forty-eight to sixty hours' confinement the susceptibility to KCN is usually again as high as or even considerably higher than that of fresh plants and the gradient is in general again basipetal. This increase in susceptibility and the reappearance of the basipetal gradient in KCN is soon followed in my experiments by the beginning of death in the apical regions.

This apparent double reversal in the susceptibility gradient and the decrease followed by increase in susceptibility to KCN requires some consideration. This case illustrates the difficulties which immediately arise when susceptibility to one inhibiting agent or condition is used as the measure of the action of another. Until we know more concerning the changes preceding death in such a case as this, it is impossible to reach any definite conclusion concerning the meaning of the changes in susceptibility, but certain probabilities suggest themselves.

In the first place the decrease in susceptibility and the more or less complete reversal of the original gradient, the same changes which have been observed in various species and under various depressing or injurious conditions (Child, '16*e*, '17), undoubtedly indicate a differential depression or injury along the axis, the most susceptible, apical regions being so much more injured than less susceptible, more basal regions, that the original relations are often reversed. The point of interest is that changes of this sort brought about by high temperature, confinement, etc., appear in the altered susceptibility gradient to KCN. This apparently means that the action of these other conditions and that of KCN on the protoplasm are not exactly of the same sort, *i. e.*, not strictly additive, for if they were, we should expect the KCN simply to continue the action of the high temperature or confinement without making them visible as a reversed gradient and a decreased susceptibility.

As death approaches, however, at least in the confinement experiments, further changes apparently occur which differ from those of the earlier stages and these changes appear in increased susceptibility to KCN and a second reversal of the susceptibility gradient, a return to the normal basipetal condition. Under ordinary conditions such increase in susceptibility would be interpreted as associated with an increase in metabolic rate, greatest in the apical regions, but it is impossible to believe that in the later stages of confinement the cells approaching death undergo any such increase in metabolic activity as these changes would indicate. It must be rather that in the later stages of the decrease in metabolism which precedes death under these conditions changes occur which are essentially like those pro-

duced by KCN so that plants at this stage behave as if they had already been subjected to KCN, and the final killing by KCN is simply or largely additive to the changes which have already occurred. The action of the ordinary narcotics such as alcohol and ether, at least in its more advanced stages, and that of KCN are also additive and thoroughly narcotized organisms are more susceptible to KCN than normal organisms, though there can be no question as to metabolic condition in the two cases. Evidently changes in susceptibility produced by one agent or condition can be made evident only by some agent or condition whose effect is not strictly additive to the first.

It seems not improbable that the present case is merely a particular example of a general relation. In terms of the permeability theory, for example, the first effect of an inhibiting agent may be, or be associated with a decrease in permeability, and if this effect is proportional to the original susceptibility along the axis, it is not difficult to understand that one such agent might produce a differential decrease in permeability and so reverse the axial gradient in susceptibility to another. In the later stages of the action of an inhibiting agent, there may be on the other hand an increase in permeability resulting from changes in protoplasmic aggregation or other changes, and after this stage is reached the effect of one agent is essentially additive to that of another. This is not to be regarded as a hypothesis in the scientific sense to account for the facts, but rather as merely a suggestion to illustrate how susceptibility relations may be altered. This case also shows certain of the limitations and difficulties of the susceptibility method in demonstrating by means of one agent the effects of another. Some of these difficulties may be removed or lessened by working with the proper combinations of agents.

For present purposes, however, the changes during the earlier, not those of the later stages of confinement are important. These changes, consisting in decrease in susceptibility and more or less reversal of the general gradient, are the same as the changes which occur very generally, though more slowly, under laboratory conditions even in running water. There can be no doubt that the transference of the plants from natural to labora-

tory conditions (Child, '16*e*), like the earlier stages of confinement, brings about a greater or less degree of physiological depression which is greatest in the most sensitive parts of the plant, the apical regions, and which, if sufficient, obliterates or reverses the original axial gradient. In cases of confinement in the small flasks as described, death usually follows this change in two or three days at most, but where the change occurs less rapidly, as in a large flask, in open standing water or in aquaria with running water, the obliteration or reversal of the gradient in the apical region is followed after two or three days by separation of the cells of the apical region in which the obliteration or reversal of the gradient occurred. In short, after obliteration or reversal of the gradient together with decrease in metabolic activity, the axis falls apart into its individual cells or into groups of two or three cells. This process begins apically because this is the region where the change first occurs, but as time goes on, if the environment does not improve, or if the plant is unable to become acclimated as a whole, the change in the gradient extends farther and farther in the basipetal direction and the separation into cells follows it in the same direction. In plants kept in open crystallizing dishes in a liter of sea-water without change, cell separation usually begins in the apical regions after several days and progresses basipetally, until after ten days most axes are completely separated into cells or small cell groups. In running water also cell separation usually begins in the apical regions after several days and progresses basipetally, though more slowly than in standing water or in a closed flask. In this case, however, separation often ceases at some level of the axis and the cells basal to this level maintain their continuity. The apical beginning, the basipetal progress, and under the less extreme conditions the halting at one level or another of this process of cell-separation constitute still another demonstration of the existence of an axial gradient in which susceptibility to these conditions is greatest apically and decreases basipetally.

This process of cell-separation is a reaction of the living cell, and is not necessarily followed by death, although under conditions in which any considerable degree of separation occurs, a varying percentage of the cells, chiefly the cells of more apical

regions, does die. In shallow crystallizing dishes with a liter of water fifty per cent. more or less of the cells may die, in more extreme conditions a higher percentage, up to one hundred per cent. in a closed flask, and under less extreme conditions a smaller percentage.

In all cases, however, where conditions are not too extreme a certain percentage of the cells may remain alive, and after a time resume growth and development. In short, these depressing conditions if not too extreme may be used as a method of obtaining isolated cells of *Griffithsia* for purposes of culture. This method is not new, for Tobler, '02, '04, has employed it for this purpose, but the fact that such cell-separation is associated with obliteration or reversal of the axial gradient is new. If, as I believe (Child, '15*b*), the axial gradient is the basis of physiological polarity, and at the same time the primary integrating factor in axiate individuation, the obliteration or reversal of the gradient must mean both obliteration or reversal of the original polarity and physiological disintegration of the individual, in other words, the elimination of the physiological order or pattern associated with and resulting from the existence of the gradient. With this physiological disintegration of the individual its component parts, the cells, become more or less completely physiologically isolated (Child, '15*b*). The fact that physiological disintegration of the individual is soon followed by physical separation of the cells is of great interest, as indicating the very direct dependence in this simple individual, of the gross morphological order upon the dynamic integrating factor.

Moreover, if the gradient is the basis of physiological polarity, we may expect that the further growth and development of these isolated cells will be influenced in one way or another by the obliteration or reversal of the original gradient. If the original gradient is completely or to a large extent obliterated in a cell, a new gradient or gradients may be established by "chance" differentials in external conditions, or may be determined experimentally. If reversal of the original gradient persists for any considerable time, the order and axiation in the further development may be determined by this factor. In the following section the development of some of these isolated cells is described, and its relation to susceptibility gradients demonstrated.

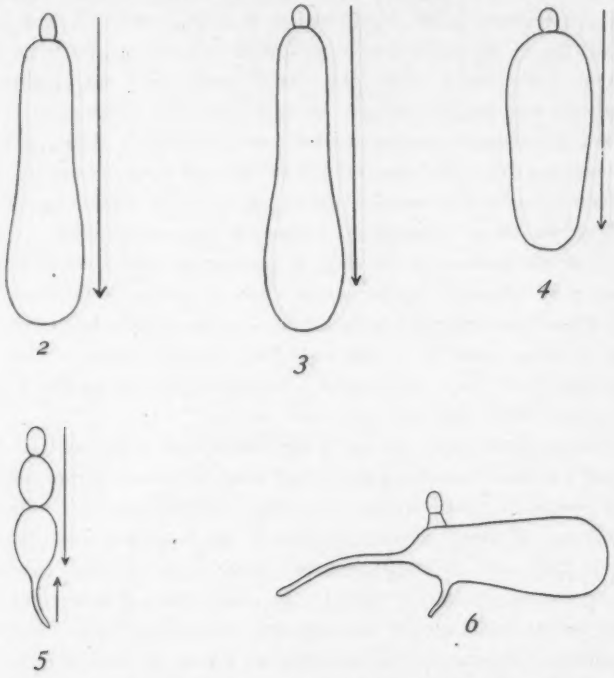
The Development of New Axes from Isolated Cells in Relation to the Gradient.

One lot of *Griffithsia*, kept in running water for three days after collection, was then placed in a shallow crystallizing dish in one liter of water and left open to the air in diffuse daylight without change of water. After two days in standing water cell separation began apically, and after seven days had extended over the whole length of the axes. Most of the cells were single, but a few groups of two, three or even four cells were observed. About half of the cells were dead at this time, the death rate being highest among the cells from more apical regions. Almost every one of the apical cells and probably 75–80 per cent. of the first three or four subapical cells were dead. Taking the shape and size of the isolated cells as an index of their approximate level in the axis, it is evident that the proportion of cells killed is greatest in the most apical regions and decreases basally, at least, to the middle regions of the axis, below which the gradient is slight, even under normal conditions. In short, the susceptibility gradient of the axis is in evidence under these conditions as well as in more rapid killing.

After another week in standing water, examination showed new growth and development in 10–15 per cent. of the living cells. Figs. 2 and 3 show cells from the more basal levels with new apical cells at what were originally the basal ends. Susceptibility gradients were determined in both these cases, the progress of death being in the direction of the arrows, the new apical cell dying before the old, large cell and the intracellular gradient in the large cell being in the direction of the arrow in both cases. In many other similar cases similar gradients were found and still other cases were observed without determination of the susceptibility gradient.

Fig. 4 shows a cell from the middle region of an axis, in which a new apical cell has arisen at the original apical end. The susceptibility gradient, both general and intracellular, is indicated by the arrow which points in the direction in which death progresses. Fig. 5 is a case in which a group of three cells from a level near the apical end have remained connected. The only new growth in this case is a rhizoid from the basal end of the

most basal cell of the group. Here the original susceptibility gradient persists in the three cells, as indicated by the arrow, while the rhizoid, like any other rhizoid, represents a new gradient in the opposite direction. The rhizoid, however, represents a



lower level of susceptibility than other parts of the individual and is the last portion to die.

In Figs. 2 and 3 the original gradient has undergone reversal, in Figs. 4 and 5 it still persists. Cases of reversal, like Figs. 2 and 3, were in general more frequent in the material than cases of persistence. This is to be expected, since cell separation is associated with obliteration or reversal of the gradient. The original gradient may persist in cells or cell-groups in which the susceptibility is for any reason exceptionally low and the differential injury consequently less marked, and probably also in cases of partial acclimation (Child, '15*a*, Chap. III., '16*d*) after

complete obliteration. It may be also that in some cells, where the original gradient had undergone reversal in connection with the basal growth, another reversal may accompany cell separation.

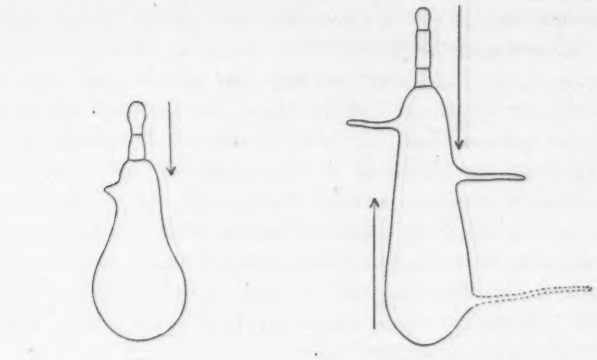
Fig. 6 shows a case in which a new polarity at right angles to the old appears in the basal region of a large cell. A new axis consisting of an apical and a subapical cell, has arisen from the lateral cell-surface near the basal end. The susceptibility gradient was not determined in this case, but there can be no doubt that the new axis represents a new gradient. The gradient in this case may perhaps have been directly determined by the differential action of an external factor, possibly concentration of oxygen or other substances necessary for metabolism. Cells lying on the bottom of the dish, or perhaps in contact with other cells, may often be subjected to such an external differential, and where the original gradient is to a large extent obliterated, a new gradient, and so a new axis may readily arise. Cases of this sort have been recorded for *Corymorpha*, among the lower animals (Child, '15*b*, pp. 142–146).

Unfortunately this culture of *Griffithsia* was made toward the end of the season, and my departure from Woods Hole prevented long continued observation. Through the kindness of Professor Osterhout, however, in turning over to me a culture of *Griffithsia* which had been in the laboratory some three months in very slowly running water, I was able to make some observations on more advanced stages of development of isolated cells. In this culture cell separation had occurred to a large extent, and many isolated cells showed considerable growth, although at the time of examination in many cases the new growths were dying or not in good condition. Figs. 7–10 show cases from this culture in which the susceptibility gradient was determined.

In Fig. 7 a new two-celled axis has arisen from the basal end of a cell, and the general susceptibility gradient, as indicated by the arrow, shows the usual relation to this axis, but the intracellular gradient in the large old cell is very slight.

Fig. 8 shows another case of a new axis consisting of three cells arising from the basal end of a large old cell and two new living rhizoids on the basal half and one dead rhizoid, indicated by

dotted lines, on the original apical region of the old cell. The arrows indicate the gradients. Death begins in the apical cell of the new axis and continues down the large cell to its middle,



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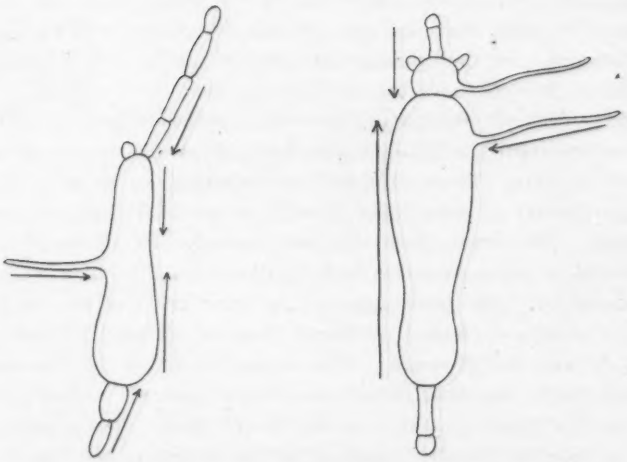

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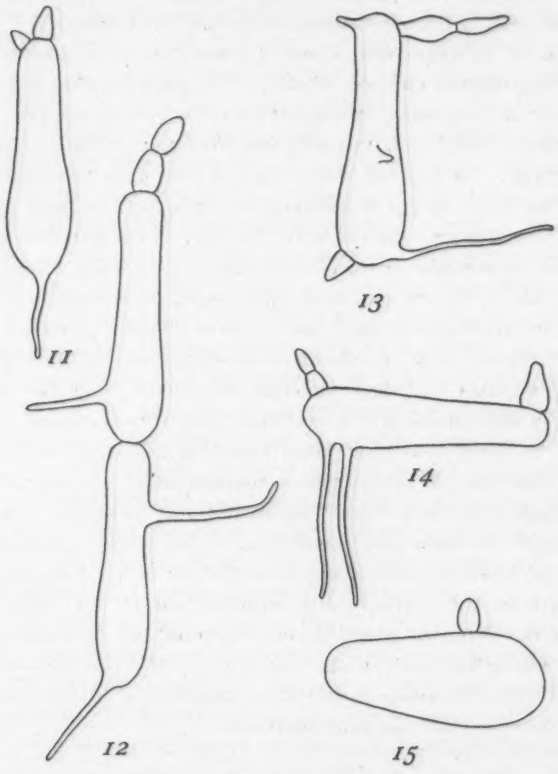
while an opposed gradient exists at the opposite end of the large cell. It is of course impossible to determine the physiological history of this cell, but appearances suggest that the cell was one of those in which the gradient had been reversed and a

rhizoid had arisen from the apical region before isolation (see p. 215). After isolation the conditions determined a double gradient, the old rhizoid died and new rhizoids arose in relation to the new axis. The original apical end of the cell, although it represents the high end of a gradient, is not active enough to give rise to a new apical cell.

Fig. 9 shows a cell with two new axes, one of four cells, the other of one cell, at its original basal end, one new two-celled axis at its apical end and a rhizoid arising from its middle region. The gradients are indicated by the arrows. It will be observed that a double gradient exists in the old cell, the middle region, where the rhizoid arises, being the region of lowest susceptibility. The two axes at the basal end and also the rhizoid were found to be more susceptible than the new axis at the apical end of the old cell, and the old cell is least susceptible of all, and its middle region the least susceptible region.

Fig. 10 is another case with new axes at both ends of the old cell. At the original apical end is one three-celled axis with two one-celled branches and one rhizoid arising from its most basal cell. At the original basal end of the old cell is another axis of two cells and the old cell also bears a rhizoid near its apical end. In this case observation of the progress of death was interrupted, and the susceptibility gradients were observed only in part. Those observed are indicated by arrows. This case showed all indications of being in poor physiological condition. The small apical cells were very heavily pigmented and instead of being rounded were flattened basally, suggesting a low turgor. The protoplasm of the large cell had also undergone structural changes similar to those which precede death in KCN and other agents. The susceptibility of all the new axes, except the one rhizoid axis arising from the old cell, was somewhat lower than that of the old cell itself. In the absence of knowledge of earlier stages in the life history of this case it is impossible to determine the meaning of the susceptibility relations between new and old parts. In several other cases the new axes were found to be less susceptible than the old cell, and in every such case the new axis, or at least its apical cell was very deeply pigmented and flattened basally, and the protoplasm of

the old cell had undergone more or less structural change. Apparently all such cases have ceased to grow and are, at least in large measure, quiescent, if not slowly dying. Their susceptibility is in general very low. Since this condition unquestionably results from the unfavorable environment, the new axes have been inhibited or injured to a much greater degree than the



old cell because of the originally higher susceptibility associated with their higher metabolic rate. If this is the case the question at once arises, why cell separation has not again occurred. As a matter of fact cell separation was occasionally seen in the new axes, though not very frequently. Its infrequency, even where

the gradient is reversed, is probably due to the greater area between the cells which is a very general characteristic of the new parts developing under these conditions. The cell turgor is apparently less than under normal conditions, and the young cells therefore depart more or less from the spherical or ovoid form characteristic of young cells produced under normal conditions, and in consequence of this change the surfaces of contact become larger and separation occurs less readily.

Figs. 11–14 show other cases of development of isolated cells from Osterhout's culture, in which the susceptibility gradients were not determined. In Fig. 11 two new apical cells arise from the original basal end, and a rhizoid from the original apical end of the cell. In Fig. 12 a new axis of two cells has developed from the basal end of a group of two cells, and rhizoids appear at various levels on the old cells. In Fig. 13 the two ends of the old cell apparently develop independently of each other, each giving rise to a new axis and a rhizoid at approximately right angles to the original axis, and a third rhizoid appears in the middle region. Fig. 14 shows a somewhat similar case with a two-celled axis and two rhizoids developed from the apical region of the old cell and a one-celled axis from the basal region. It will be noted that in all these cases the new apical cells arise at or near one or both ends of the old cells. Among all the cells examined, both from Osterhout's and from my own culture, only one case was found (Fig. 15) in which a new apical cell arose from the middle region of the old cell. This case was returned to the culture after examination in the hope that further development would occur, but could not be found later. Numerous other cases of development occurred in the isolated cells of these two cultures, but those described give a fair idea of the variety of axial relations observed.

Discussion.

If the axial susceptibility gradient is in any degree an indication of the general metabolic relations along the axis, and if these metabolic relations are effective factors in determining the axial order or pattern, then the apical cell represents the region of highest metabolic rate, and in the vigorous axis under good

environmental conditions a gradient of decreasing metabolic activity extends basally. The rhizoid itself is a similar gradient, and the internal condition which favors rhizoid-development is apparently a low metabolic rate, though external conditions may determine whether it actually develops or not. It is, in fact, like the stolon of the hydroid, *Tubularia*, a secondary gradient, originating as a local region of increased metabolic activity in consequence of partial physiological isolation (Child, '15*b*, pp. 91, 92), in which external factors probably, at least often, play a part (Child, '15*b*, pp. 132–134). The rhizoid, in short, represents a secondary, more or less subordinate gradient, originating at what may be called a certain metabolic level in the axis or between certain metabolic limits and probably developing in relation to certain external factors. This conception agrees well with the fact that the susceptibility of the rhizoid, even at its tip, is usually lower than that of the apical region of the main axis, while even at its base, its susceptibility is usually higher than that of the level from which it arose.

Rhizoid-formation is not necessarily limited to the extreme basal end of the axis, for as metabolic rate decreases with advancing age of the cells, other levels may attain a condition which permits rhizoids to develop, and rhizoids may therefore appear first at the base and later rhizoid-formation may progress acropetally along the physiologically older portions of the axis, as it actually does in various species of algæ (Tobler, '06) and to some extent and rather irregularly in *Griffithsia*. As regards their relations to the primary axial gradient the roots of the higher plants are probably similar to the rhizoids (Child, '15*b*, pp. 156–163), though their function is of course different.

Assuming the correctness of this conception of the simplest plant axis, we should expect to find that in cells isolated from a preëxisting axis new apical cells would arise from the region of the cell possessing the highest metabolic rate and rhizoids from the region of lowest rate, or at least from lower metabolic levels than the apical cell. So far as determined, the susceptibility gradients in the forms developed from isolated cells agree in general with this expectation. In every case from my own culture, where only the earlier stages of new axial development

were present, the new apical cell arises from the region of highest susceptibility in the old cell. In the more advanced stages of development in the Osterhout culture most of the cases in which the susceptibility gradients were determined show the same relations, but some exceptions occur, apparently in cases which are in poor condition or gradually dying. Moreover, the fact that in these isolated cells new apical cells arise more frequently from the basal end or from both ends of the old cell than from the apical end alone is undoubtedly connected with the fact that cell separation is associated with obliteration or reversal of the original axial gradient in the cells. The greater frequency of development of new apical cells at or near one or both ends of the old cell than in the middle region is probably also an expression of gradient relations. Where the intracellular gradient was primarily basipetal and is completely reversed, the basal end of the cell becomes physiologically apical, and morphological development follows the new metabolic relations. Where the original gradient is largely obliterated, the two ends of the cell which were the regions of connection with other cells are doubtless more affected than other parts of the cell by the change in conditions accompanying the separation and undergo a certain amount of regression and rejuvenescence, and so attain a higher metabolic rate (Child, '15*a*, Chap. X.). It is also possible that in some cells from the more basal regions, where secondary basal elongation is occurring, and where two opposed intracellular gradients are often present (see p. 215) before separation, the obliteration of these gradients is not complete at the time of separation, and the higher rate at the two ends is still further increased by separation, so that both ends become physiologically apical. And finally, where the gradient is reversed in the normal plant, as sometimes in the more basal cells, it may be again reversed, obliterated or almost obliterated at the time of separation with correspondingly different results in later development.

While it is impossible to follow the changes in the gradient in a particular cell and to correlate them with the later development, the high degree of constancy in the relation between the intracellular gradients and the localization of the new axes leaves

little room for doubt concerning the fundamental character of the relation.

The cases where the new axes arise from the lateral surface rather than the end of the cell, as in Figs. 6, 13, 14, or from the middle region, as in Fig. 15, cannot be definitely accounted for, but are doubtless connected with special external conditions, perhaps the position of the cell with respect to light, oxygen supply or other external factors, or perhaps with local metabolic differences in the cell produced in some other way. I believe, however, that if we could analyze these cases, we should find that they, like the others, are an expression of the existing gradient relations, however these may have been determined (cf. Tobler, '06).

The localization of the rhizoids also agrees in general with expectation as far as the data go. In general they arise from a relatively low metabolic level on the cells, but this level may be in the middle region (Fig. 9) or at or near one end (Figs. 5, 6, 11), or rhizoids may appear progressively over a considerable length of the cell or cells (Figs. 8 and 12).

As regards the facts of morphological development, these experiments add nothing of fundamental importance to those of Tobler ('02, '04, '06), but the determination of the relations between the susceptibility gradients and cell separation and the localization of new axes serves to throw some light on the physiological conditions concerned in this development and on the problems of physiological polarity and physiological integration or individuation.

These experiments suggest further possibilities of more exact control of the localization of new axes on isolated cells, such as the determination of the influence of the direct subjection of single cells to the differential action of external factors on the localization of new axes.

Summary.

1. The original axial susceptibility gradient of the alga *Griffithsia* is more or less completely obliterated or reversed after one or two days of confinement in a small volume of water, and the larger the volume of living plant protoplasm introduced, the more rapid the changes.

2. Where the conditions of confinement are extreme, obliteration or reversal of the gradient is followed in one or two days by death with a basipetal gradient, but under less extreme conditions the alteration of the gradient is followed by the separation of the axis into individual cells or occasionally small cell groups, the separation beginning apically and progressing basipetally.

3. Obliteration or reversal of the original axial gradient, followed by cell separation occurs, though more slowly, when the plants are kept in open standing water and to a greater or less degree, but still more slowly, in slowly running water in diffuse daylight.

4. A variable percentage of the isolated cells dies, the death rate being in general highest in apical cells and decreasing with the level of the cell in the axis, at least to the middle levels, below which there is often little difference. The cells which do not die may undergo a new development, giving rise to new apical cells and so to new axes.

5. Determinations of the susceptibility gradients in these reproductive developments from isolated cells show that the new apical cell arises from the region of highest susceptibility in the old cell. This region is more commonly basal than apical, because the gradient is usually reversed at the time of cell separation, but both ends of the cell may give rise to new apical cells where double opposed gradients exist. Rhizoids, on the other hand, arise from low levels in the axial gradient.

6. These experiments show that the original polarity of the cells of *Griffithsia* can be completely obliterated or reversed, and that the morphological development of new axes is an expression of gradient relations present in the cell at the time.

7. The facts support the conclusion that a gradient in metabolic rate, protoplasmic condition, or whatever we prefer to call it, of which the susceptibility gradient is within certain limits an indicator, constitutes physiological polarity in protoplasm, and that such a gradient is not an inherent property of protoplasm, but may be determined and altered by external factors.

HULL ZOÖLOGICAL LABORATORY, UNIVERSITY OF CHICAGO,
January, 1917.

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THE EFFECT OF ETHER AND CHLOROFORM ON CERTAIN FISHES.[1]

HAROLD D. CLAYBERG.

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I. INTRODUCTION.

Various drugs which cannot be standardized chemically must be standardized biologically before being used for medical purposes. The time required to kill an animal (for example, a goldfish) has been used for this purpose (see particularly Pittenger and Vanderkleed, 1915). The discussion about digitalis has been particularly keen. The effect of a large series of coal tar derivatives upon various wild fish has been studied by Shelford ('17) in connection with stream pollution work. He determined their relative toxicity by killing specimens of the orange-spotted sunfish. The work described below was undertaken with the two common drugs, ether and chloroform. Their effect on the orange-spotted sunfish and on certain other wild species was studied in the hope that it might serve to relate the coal tar results with those of the investigators interested in the standardization of drugs with domestic goldfish.

It is the purpose of this paper to point out that there seem to be three possible ways of standardizing a drug with fish.

1. Type of behavior in a gradient between pure water and water plus the drug being tested might be used. To furnish

[1] Contribution from the Zoölogical Laboratories, University of Illinois, No. 90

definite standards a distinct form of behavior should appear or disappear at a definite concentration.

2. Time till definite symptoms of sickness appear might be used. Such a condition for serial concentrations is shown to the left in chart II.

3. Killing time is the only standard thus far applied.

In connection with killing it is our purpose to point out that the time-concentration curve is probably an hyperbola which, if fully demonstrated for a few substances, will render the investigation of drugs in general much easier.

II. Reactions.

1. *Materials and Methods.*—The reaction experiments were performed in the Shelford gradient tank. This is a tank 122 cm. long by 15 cm. wide by 13 cm. deep. Water is allowed to flow in at each end at a definite rate. It enters the tank through two tees, the cross bars of which are perforated with small holes so as to distribute the inflow throughout the width of the tank. It flows out at the middle at both top and bottom so the two kinds of water meet at the center. In these experiments normal water was run in at the left end at a rate of about 450 c.c. per min. and normal water plus ether or chloroform was run in at the right end at an equal rate. The drug solution was made up in an 18-liter bottle, placed above the apparatus, and siphoned out. Siphon and bottle were closed on account of the volatility of the poisons used. To be certain that the concentration under observation remained constant, the flows were tested occasionally throughout the experiment; hence the concentrations quoted are presumably accurate. The outflow at the center did not prevent the mixing of the two kinds of water and thus the middle section equal to one half or third of the tank, was a gradient between the normal water and water plus ether or chloroform. Accordingly as a fish moved from left to right in the tank it encountered a gradually increasing concentration of the poison. We found no evidence that fish react to the slight current through the tank. Since each half of the tank held about 9 liters, it required 21 minutes to fill it or to replace all the water in one of the halves.

Two 8 candlepower electric lights were fixed above the center of the two halves, *i. e.*, above a point midway between the screen partition in front of the introducer tees and the center drain. The lights were 15–20 cm. above the surface of the water which was 13 cm. deep. The tank and lights were enclosed under a black hood. The experiments were observed through openings in the hood above the lights or through the glass side late at night. Water differing as little as possible from that in which the fish usually live was used for control readings. Controls were observed and the conditions in the two ends of these were the same either because the water introduced at the two ends was alike or because no water was run into either end (standing water). Behavior of both bullhead and sucker in tank controls was of two types identified as *quiescence* and *circuiting*. The latter refers to swimming around the tank and involves both back and forth movement and crosswise movement. The crosswise movement, while ignored in the charts, should be understood to occur, especially in the case of controls. In controls quiescence predominated after a short period of circuiting (see Chart I., Bullhead Control).

In experiments of this kind the reactions of the fish are commonly graphed, that is the distance between two lines on a sheet of paper (see chart I.) is used to represent the length of the tank. Cross movements of the fish are ignored in the graphing. Vertical distance is used to represent time according to scale. Thus in chart I. control 3 the bullhead went the entire length of the tank twice during the first one minute and 20 seconds.

For the reaction experiments only suckers (*Catostomus commersonii*) and bullheads (*Ameiurus nebulosus*) were available, but their behavior is not materially different from that of the standard sunfish (*Lepomis humilis*). More fish were not obtainable on account of the swollen condition of the streams, hence the small number of experiments done.

2. *Reaction and Standardization.*—Standardization through reaction in gradients would be dependent upon (*a*) Definiteness of some change in reaction occurring (in case of the fish); (*b*) accuracy of the change with reference to concentration; (*c*) speed of standardization; (*d*) reasonably low cost of the process.

CHART I.

Graphs of Reaction of Fish to Chloroform and Ether.

(See Table I.)

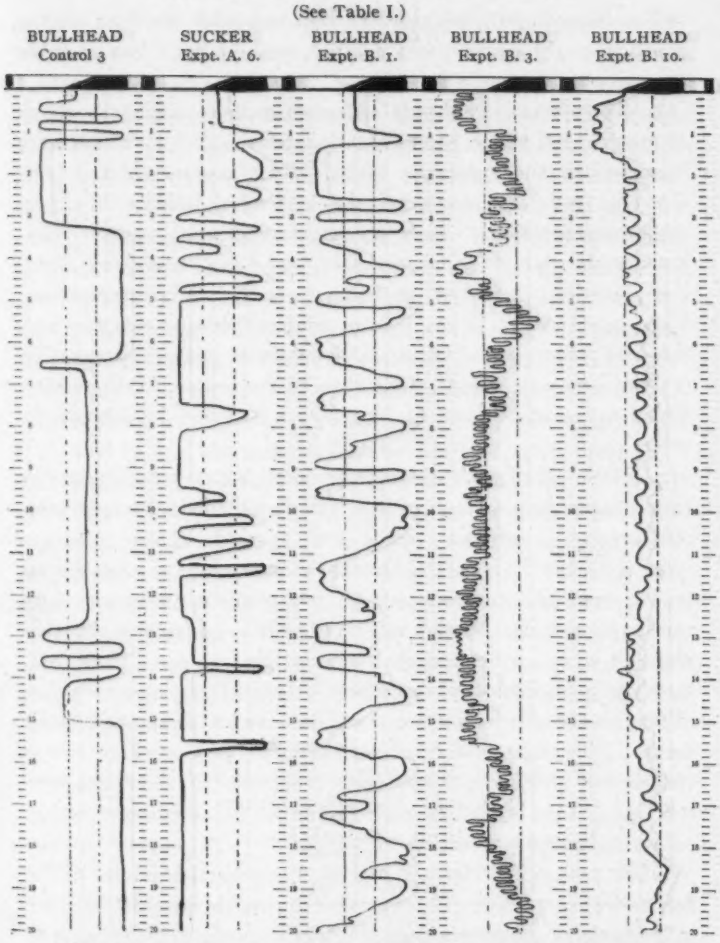

CHART I. Reaction graphs. The drug was introduced at the right; its concentration is indicated by the larger black area in the heading. These graphs represent parts of experiments only and are meant to show, primarily, the type of action of the fish to certain poison concentrations in the gradient tank. To the extreme left is shown a control experiment on a bullhead and it should be compared with the three to the right (of series B) in order to bring out the distinctive types of behavior involved. Not considering the sucker experiment and taking only those shown on the bullhead it is plain that an intergradation of action can be traced from the one on the extreme left to the one of the extreme right.

The reaction of the sucker to chloroform is shown in the second graph to the left and should be compared with the reaction of the bullhead to ether as shown in Expt. B. 1 for the concentrations are approximately isotoxic; the bullhead showing notches which are never present in the charts of suckers.

The following studies were carried out with the idea that a reaction might prove more definite, and perhaps less variable than time of death.

3. *Chloroform.*—Bullheads and suckers were used in a series of experiments which began with low concentrations which were increased in the successive trials. Both species showed considerable individual variation and in general failed to react to most concentrations. Both species showed some positive reactions in the case of .14 c.c. per liter (213.8 mg.) and a few negative reactions occurred in somewhat higher concentrations. There appeared to be no definite relation between reaction and fish size. The general failure to give definite positive or negative reactions may be due to a paralysis of the sensory endings with which fishes are known to recognize substances in solution in water.

4. *Ether.*—Bullheads alone were used, but a complete series of ten experiments were run in which concentration ranged from .267–4.573 c.c. per liter (192.24–3292.56 mg.). These solutions were all distinctly non-fatal because no symptoms of even partial loss of equilibrium were observed. Although some experiments ran for three hours, the fish exhibited no true positive or negative reaction when any experiment as a whole was considered. If, however, a small piece of one were carefully chosen an impression of reaction in either direction could be obtained from any experiment. The piece chosen from experiment B. 3 on chart I. was chosen for another purpose but it appears to show negative reaction. This is not the case as the whole experiment rather points to indefinite reaction.

While tank end preference was nil, the general behavior of the fish underwent definite change twice during the gradual increase in strengths of the poison used. Chart I. contains critical experiments of which repeated mention will be made through the rest of the reaction discussion. Experiment B. 1 was the first of the series and shows that circuiting predominated; but occasional little notches occur in the curve that are absent in the sucker's curve (exp. A. 6) and which are very suggestive of the behavior of the bullhead to higher concentrations. Thus experiment B. 1, having a concentration of .267 c.c. per liter (192.2 mg.), fixes a

definite sort of behavior for comparison with later experiments.

After 100 minutes in this .267, or after 13 minutes in .403 (290.1 mg.) a very distinct third type of reaction appeared. This might very appropriately be named the *nosing reaction.* It was characterized by incessant restlessness and continual change of direction; the fish nosed back and forth in a peculiar uneasy manner, turning around 180° every 2–8 seconds, holding its head always close against the glass side of the tank, and keeping this up for hours. Experiment B. 3 shows how remarkably this reaction differed from the others. In concentration 4.573 (3,292.5 mg.) this nosing reaction was entirely eliminated, and a still different type of reaction appeared (see exp. B. 10).

This reaction might well be designated as *intermediate,* since it seemed to be a compromise between the circuiting of exp. B. 1 and the nosing reaction of higher concentrations, in that it exhibited the slow turning of the former combined with the narrow range of the latter. The range of the nosing reaction in relation to concentration is shown in Table I. It seems that such an apparently precise reaction ought to be found with fish or other aquatic animals in the case of drugs needing standardization. Comparison of Table I. with parts of three experiments shown to the right in chart I. will make clear the significance of the results attained.

TABLE I.

SHOWING RELATION BETWEEN ETHER CONCENTRATION AND TIME TILL INCEPTION OF THE NOSING REACTION IS OBSERVED.

Series B. Exp. Number.	Poison Entry Rate in Cu Mm./Sec.	Poison Concentration at Right End of Tank.		Time till Nosing Reaction Started, Minutes.	Duration of Expt., Minutes.
		C.c. per Liter.	Mg. per Liter.		
1	2	.2667	192.0	100	128
3	3	.6667	480.0	30	182
5	10	1.3333	960.0	10	86
9	30	3.7855	2725.6	0	97
10	35	4.5733	3292.6	*	72

*No nosing reaction whatever occurred throughout this experiment.

III. RESISTANCE.

1. *Materials and Methods.*—Killing of fish was carried on in closed bottles in which poison solutions of known concentrations were made up. As a rule 4-liter bottles were used, although

some of 2- and 5-liter were also utilized. The fish was placed in the solution after being measured and weighed; and the time till its behavior became definitely abnormal and the time till death were carefully noted.

2. *Results with the Minnow* (*Pimephales*).—Preliminary work was undertaken looking towards a comparison of the closed bottle method of killing (used in all drug standardization work) with that of running poison of known concentration at definite rate through a bottle. The results indicated that the two methods had like effect for the same poison concentrations. Further, the results showed that the hour killing concentration for the minnow was between 2.2 and 2.3 c.c. per liter for ether (1,584–1,586 mg.). Weight used varied from 1.5–4 grams.

3. *Ether: The Sunfish.*—The standard used by Shelford for the measurement of toxicity of poisonous polluting substances is the concentration of any poison sufficient to kill a 4–5 gram *Lepomis humilis* in approximately 60 minutes. The hour killing concentration for ether was found to be between 3.685 and 3.690 c.c. per liter (2,653.2–2,656.8 mg.). Apparatus at hand permitted no closer determination. The number of experiments run was sufficient to plot the death curve shown in chart II. The base line or X-axis was used for minutes until the fish became abnormal or dead. The Y-axis was used for the concentrations employed. Points for all experiments were plotted and these were connected to form a curve. This curve showed a certain width due to variation in time of death throughout, which has been brought out by stippling.

The data were now separated into three classes to enquire into the effect of fish weight on death time. Since not enough fish of strictly standard size could be obtained, fish of size between 2 and 25 grams were used. Those obviously outside the weight from external appearance were used to determine the critical concentration roughly and those nearest the proper standard were used last. These were now placed in three classes: class 1, 2–9 grams; class 2, 10–19 grams; class 3, 20–25 grams. These were plotted in three curves on chart II. Those of classes 1 and 2 were complete enough to show the following facts.

At about 4.8 c.c. per liter (3,456 mg.) the fish of the two classes

CHART II.

Death Curve of Lepomis Humilis in Ether.

($(C_2H_5)_2O$.)

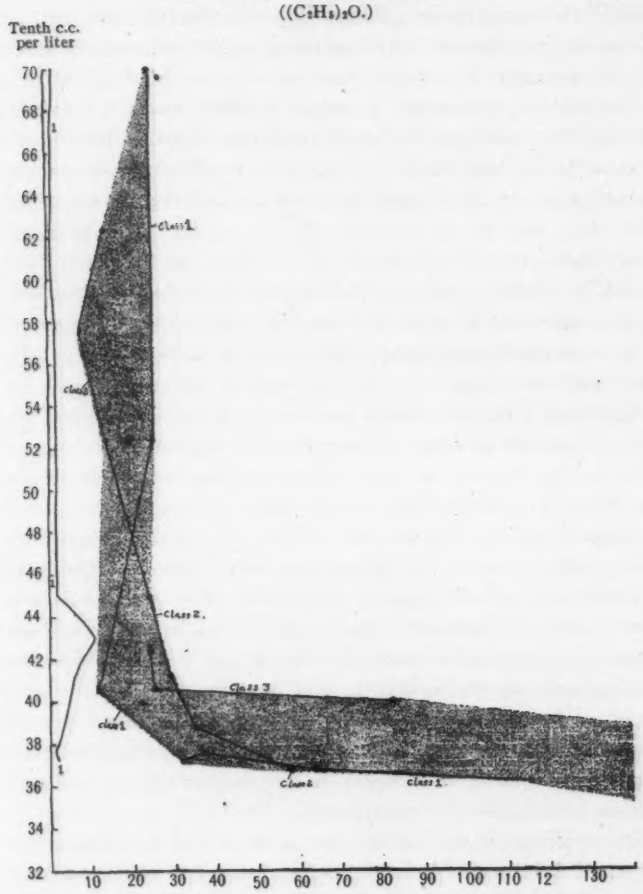

CHART II. (Ether death curve.) The curve to the left is the "abnormal curve" and marks the inception of symptoms such as loss of balance. The obvious fall in toxicity below 420 cu. mm. per liter is shown by the stippled death curve turning off to the right. Class 1 was of fish 2–9 grams in weight; class 2 was of fish 10–19 grams in weight; class 3 of fish 20–25 grams in weight. The significance and curve directions are discussed in the text. It is very likely there would have been much intercrossing if many experiments had been run and ten or more classes had been separated. It may be that difference of physiological condition of different batches of fish (as chance allots in the purchase of fish for standardizing) may give results with the same concentration of the same drug as far apart as the different weights of fish here have done. In the case of the fish used here all were caught in the same place, and kept in constant environment in the same tank until used.

The upper and lower boundaries of the curve at the lower right hand edge of the cart were determined by experiments not shown in the graph. I am indebted to Miss Dorothy Wing, of the University of Chicago, for aid in the preparation of this chart.

died in the same time. Above that concentration the toxicity of the poison increased for the 10–20 gram fish and decreased for the 2–9 gram fish up to about 5.3 c.c. per liter (3,816 mg.), beyond which it remained constant. Below about 4.8 c.c. per liter, on the contrary, the toxicity of ether increased in the case of class 1, and decreased in class 2 to a maximal difference at about 4.1 c.c. per liter (2,952 mg.) below which the two converged until they met at 3.7 (2,664). Their crossing point is at the hour death concentration. If, at certain points, the death curves of different weights of fish are found to cross, in standardizing aconite, for example, it would be well to take these points as the standard and disregard the hour standard or any other previously set.

The third class furnished complete data only between 4 and 4.35 c.c. per liter (2,880 to 3,132 mg.). At the upper concentrations the toxicity is intermediate between classes 1 and 2. But below 4.1 c.c. per liter (2,952 mg.) the toxicity falls away far more rapidly than for the others. It is very likely that concentrations below 3.9 (2,808 mg.) will cease to have toxic effect of any sort on class 3. Since the critical concentration is well below this point the significance of weight of the fish used is seen to be of the utmost importance. It is regrettable that no large fish were available to test out the end of the class 3 curve. Very small fish below 2 grams are, as might be expected, killed in much less time than classes 1 or 2. Thus the resistance of the fish is seen to be consistently different for different weights and also of considerable complexity.

The crossing of the curves of classes 1 and 2 renders both classes of data available in the determination of the hour death point. The above results point out that, in standardization of drugs by fish, the different sizes may well prove to differ consistently and considerably. For this reason it would seem that the weight problem needs careful analysis and extensive experimentation to determine the proper weight limits to be allowed for results of the accuracy demanded.

Examination of the fish led to the graphing on the same sheet (see chart II.) of the time it took the fish in any concentration to become definitely abnormal (loss of balance, etc.). Above 4.5

c.c. per liter (3,240 mg.) the time was 1–2 minutes but below this concentration there occurred either a sudden decrease in initial toxicity or an equally strong increase in the resistance of the fish. This culminated at 4.3 c.c. (3,096 mg.) per liter in an "abnormal" time of 11 minutes, and from there, with further *decrease* in strength of solution the initial toxicity *increased* to the norm (1–2 min.) at a concentration of 3.7 c.c. per liter (2,664 mg.).

If a relation such as this be found for fish in standardizing some drug, it will be both quicker and cheaper to use it instead of death. Decreased initial toxicity with decrease in strength and close approach to hour killing concentration would be in no wise remarkable if it did not increase again to the norm as the concentration fell to the hour killing concentration as determined. But enough fish were used to render this inexplicable phenomenon reasonably certain.

4. *Chloroform: the Sunfish.*—The series run here were also plotted as explained for ether. See chart III. The hour death concentration lies between about .07 and .1 c.c. per liter (106.89 and 152.7 mg.) and much variation is exhibited between these points. The rest of the curve shows no such variation but it may be that this was due to the small number of experiments used to determine them. It is equally likely that only certain limited regions exhibit variability. Should this be the case in a drug needing standardization the variable areas should be located and no standard set anywhere within one of them. Continuous variation throughout a curve would, if wide enough, forbid the use, for accurate standardization work, of the experimental animal used in making the curve.

IV. Comparison of Ether and Chloroform Killing Curves.

It has been stated that these curves are hyperbolæ or very close to them. It should be further noted that the maximum death variability seems to coincide with that part of the curve showing greatest curvature. The laws governing hyperbolæ will probably be found to be of great use in fish standardization since they seem to die according to one. Twenty-four experi-

CHART III.

Death Curve of Lepomis Humilis in Chloroform.

($CHCl_3$.)

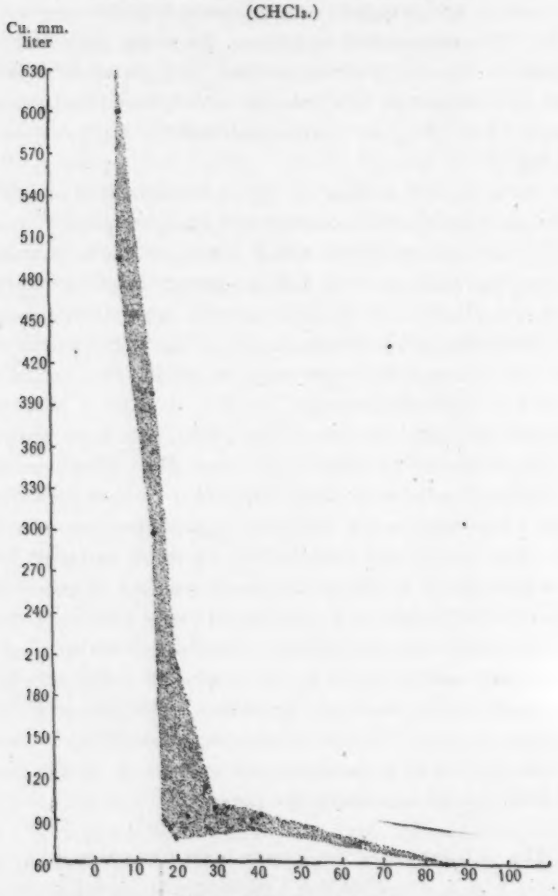

CHART III. (Chloroform death curve.) Each point represents a separate experimental result. The poison is obviously very toxic as a concentration of less than .09 c.c. per liter kills in an hour. There is a sudden change in the curve between the concentrations of 100 and 200 cu. mm. per liter as if a new factor suddenly entered into the determination of the curve below the limits indicated. Only four of the fifteen fish used were above 6.2 grams and these were all used to determine the curve above 190 c. mm./liter. Hence no classes by weight could be separated.

ments were used to determine the ether curve and 15 for that of chloroform. This does not seem to be sufficient data to establish a law although it appears obvious that there may be one here. The upper limb of the chloroform curve is nearly a straight line and in it $c \times t = k$, about 4,200, c being concentration in cu. mm. per liter and t being time until death. It is probable that time, weight of fish, and concentration of poison used, will prove the determining factors for relative toxicity.

The similarity of the chloroform and ether curves suggest that after the location of a toxic zero, Krogh's ('14) Law may be found to govern under certain limitations. Should a law be here discovered and prove to hold closely enough, it is easily seen that it will prove of very great value in the standardization of poisons by means of fish.

V. Poisoning Symptoms.

1. *Ether.*—No particular irritability or spasmodic movements were noted. The fish at first acted normal, then swam vertically with head up, lastly quieted down and lay on its back or side, very usually at the surface of the water.

2. *Chloroform.*—For anæsthesia with either chloroform or ether Cushny[1] notes three stages: (1) Imperfect consciousness, (2) excitement, (3) anæsthesia. In fish, although the first stage is almost impossible to distinguish from the normal, the others may be definitely noted. The second stage is characterized through all concentrations used by a variable period of violent muscular contraction, which throws the fish wildly in all directions so that it bumps its nose strongly on the glass each time. Each bump is followed by a motionless pause of one half to one second. The third stage, coma, is reached in a time increasing as the concentration decreases. The fish lies on its side, or rarely on its back, at the bottom of the jar and the opercular movement is regular at first, then intermittent, and finally ceases. While the fish respires regularly it may recover if held under a stream of running water.

3. *Comparison of Ether and Chloroform.*—(*a*) In ether the stage of excitement is short or omitted while in chloroform it is

[1] Pharmacology, p. 151.

obvious and protracted. (*b*) In the sunfish ether produces good anæsthesia allowing easy recovery, while chloroform poisons rather than anæsthetizes. (*c*) Honigman[1] states that with man chloroform is approximately three times as strong as ether (by weight). With the sunfish it is about 44 by volume or 20.4 by weight. (*d*) From previous statements it might be well to add that, if death remain the biologic change used for standardization, in certain cases it may be that other killing times may prove more accurate than the 60-minute standard.

4. *Comparison of Ether and Chloroform with Coal Tar Derivatives.*—Shelford ('17) has investigated the effect of various coal tar derivatives on standard fish. In comparing these poisons with the drugs previously discussed in this paper the following method was used. The poisons were put up in concentrations twice as strong as would be necessary to kill in an hour with the object of killing the fish in approximately 15 minutes. A specimen of golden shiner (*Abramis chrysoleuca*) was put into a 2-liter bottle of each poison solution and when it was dead a second was inserted. The actions of the fish were noted carefully. In all cases the opercular movement was greatly increased in amount and rate at the beginning of stage 2 (excitement) and later weakened and decreased. The action of these coal-tar derivatives as irritants and poisons was like that of chloroform but distinctly different from that of ether. A large amount of blood appeared to collect at the ventral side of the body toward the end of stage 2. This was notably so in quinoline, less so for aniline and toluene, and observable in the others. No such phenomenon was observed in the parallel chloroform and ether bottles.

TABLE II.

Poison Used.	Concentration in Cu Mm./Liter.	Time Different Fish Were Killed In.	Relative Irritability.	Opercular Rate Range Observed During Experiment
Toluene.....	150	15, 23	Highest at first.	60– 88
Quinoline.....	100	12, 20	Longest.	27–144
Aniline.....	2,000	8, 9, 10	Very strong.	30– 60
Orthocresol.....	100	47	Medium.	70–240
Chloroform.....	600	7, 18	Strong.	22–180
Ether.....	4,250	18, 20	Very little.	90–102

[1] Cushny, *op. cit.*, p. 163.

VI. Summary and Conclusions.

1. The behavior of bullhead and sucker in gradient tank to chloroform was usually indefinite and followed no rule.

2. Bullheads exhibit a peculiar nosing reaction in ether concentration (in gradient tank) between .3 and .4 c.c. per liter but not above or below these limits (except as noted). Such definite and remarkable changes in behavior may be determining factors in use of fish for standardization of poisons.

3. Concentrations of about .07 c.c. (106.89 mg.) per liter chloroform or of 3.69 c.c. (2,656.8 mg.) ether can kill standard sunfish in an hour.

4. In ether during third stage of anæsthesia the fish usually lies belly up at the surface while in chloroform it tends to lie on its side at the bottom of the bottle.

5. Toxicity ratio between chloroform and ether is 3 : 1 (by wt.) for man and 20 : 1 (by wt.) for the sunfish. Such constants may be of critical value in comparing the relative usefulness of different animals for standardization uses. It may not be the animal nearest man as regards toxicity ratio which is most valuable for standardization. For one where the toxicity ratio is far greater, and hence an animal far more susceptible, may be most accurate in perceiving and reacting to, or in dying from, the poison demanding measurement. Nearness or likeness to man is of little account and precision of reaction or death the paramount issue.

6. Ether anæsthetizes the sunfish while chloroform acts more as a poison, being, in a certain way, intermediate between ether and the violently irritating coal-tar derivatives studied above and in Shelford's work.

I take this opportunity to express my thanks to Professor Victor Shelford, of the University of Illinois, for the courteous help and kindly encouragement which made this paper possible.

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THE HISTORY OF THE CHROMOSOMAL VESICLES IN FUNDULUS AND THE THEORY OF GENETIC CONTINUITY OF CHROMOSOMES.

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At the Columbus meeting of the American Society of Zoölogists in 1915, the writer reported the occurrence of chromosomal vesicles which could be recognized as persistent entities even in the resting stage. Since that time it has been possible to trace the history of these vesicles from the metaphase condition of the chromosomes through the anaphase, telophases and the various stages of interkinesis back to the prophase stage where the new chromosomes are fully formed as new bodies which are as distinctly chromosomal in character as the more familiar ones of the metaphase. This history can now be given with but few gaps; and these are of comparatively little importance. The bearing of these facts on the hypothesis of genetic continuity of the chromosomes is direct and very important.

The presence of persistent chromosomal vesicles was first noted in the study of the eggs of *Fundulus heteroclitus* which had been fertilized with the sperm of *Ctenolabrus* (*Tautogolabrus*) *adspersus*, and the history was first made out in a general way upon those cleavage nuclei. Subsequently the observations were extended to the *Fundulus* eggs fertilized normally by the sperm of the same species, and the gaps in the series filled in from this form. So far as could be determined there is no essential morphological distinction in the figures presented by the normal and the hybrid nuclei. The same account applies to both, with the exception of certain points to be noted later, which do not apply to the general facts and line of argument here given.

The first observations were made while working on the hybrid eggs to determine the effect of radiation upon the development, particularly with the reference to the chromosomes which differ in form and which can be recognized as distinct in the hybrid eggs as

was shown by Miss Morris following the lines of the classical work of Moenkhaus.

In 1904 Moenkhaus's well-known paper appeared announcing the independence of two chromosome groups on the spindles of dividing eggs of *Fundulus* and *Menidia* hybrids, and the recognition, based on morphological evidence, of the two groups as from the male and female parents respectively. Conklin (1901) had already recognized the male and female halves of *Crepidula* cleavage nuclei; Ruckert, (1895) and Häcker (1895, 1902) had seen the double nuclei and bilateral distribution of chromatin in *Cyclops;* and Herla (1893) and Zoja (1895) had traced the independent maternal and paternal chromosomes of *Ascaris* eggs to the twelve-cell stage. Moenkhaus, after identifying in the normal eggs the chromosomes of *Fundulus* as straight rods and those of *Menidia* as shorter curved rods, easily distinguished the male and female contributions to the cleavage nucleus of the hybrid eggs.

Since then a great deal of work has been done on the hybridization of many animals, and some further studies have been made on the crossing of different species of fish. In this group of animals, however, cytological observations have not been much extended. Yet G. and P. Hertwig figure a double spindle showing the portions derived from the two parents, and Miss Morris has corroborated Moenkhaus's principal findings in her crosses of *Fundulus* and *Ctenolabrus*. In the latter case, the *Fundulus* chromosomes, which are small straight rods, as Moenkhaus found them, differ clearly from those of *Ctenolabrus*, which, according to Miss Morris, are small and round.

At Woods Hole[1] in the summer of 1914 I repeated these experiments of Miss Morris on crossing the eggs of *Fundulus* with *Ctenolabrus* sperm. Since the reciprocal cross is not easily made, and since the fact as to whether the cross is possible was not under investigation, my experiments were limited to crossing in the one direction. It had occurred to me that some light might be thrown on the question of the effects of radioactivity on chromatin by treating variously with X-rays the eggs and sperm

[1] I am greatly indebted to the Director of the Marine Biological Laboratory for his kind assistance in providing me with the facilities of the laboratory.

in these crosses where the chromosomes in the two differ. Experiments in four series with controls were therefore set up, and were treated with long and short exposure to X-rays as follows (adopting the series designations used by the Hertwigs in their radium experiments). Radiation treatment is indicated by *r*; untreated, or normal eggs and sperm are represented by *n*.

Controls. $nF\,♀ \times nC\,♂$ unradiated.
Series A. $nF\,♀ \times nC\,♂$ radiated after fertilization.
Series B. $nF\,♀ \times rC\,♂$.
Series C. $rF\,♀ \times nC\,♂$.
Series D. $rF\,♀ \times rC\,♂$.

In general my observations corroborate those of Miss Morris as to the regular course of the events in the hybridized eggs of *Fundulus* and *Ctenolabrus*, and I find very little divergence in the behavior of the definitive chromosomes here from that described by Moenkhaus for *Fundulus* and *Menidia* crosses.

In general also, my results are in line with those of the Hertwigs in their radium experiments as to the injurious effects of the radiation upon the chromatin, shown, for example, in the failure of the egg nucleus in the series C, when strongly radiated, to take part in the development of the egg, as can be determined by the kind of chromosomes present on the spindle. Similarly, strongly radiated sperm chromatin in series B is killed and takes no part in development.

In the course of the investigation upon the radiated eggs a set of facts was discovered which bear directly upon the ever recurring question of chromosomal continuity, and which seemed to the writer, worth following out. It is the purpose of this paper to set forth those findings, and to show their bearing upon the important problem of cell structure, and to give at this time the results of the radiation experiments only very briefly, leaving them to be presented elsewhere.

During the early stages of the investigation the material studied was chiefly that which had been radiated, and most of the drawings given are from those slides. Subsequently, however, eggs of *Fundulus* which were unradiated have been sectioned, and studied from the same point of view and the conditions ascertained to be exactly the same as far as the formation

of the chromosomal vesicles and their history is concerned. I wish to emphasize that neither the fact of the hybridization in the *Fundulus* ♀ × *Ctenolabrus* ♂ crosses nor the exposure to the X-radiation is responsible for the conditions here figured, for a full set of figures from normal *Fundulus* eggs in addition to those here presented could easily have been made and exactly the same conditions depicted, had the differences been thought to warrant the extra effort and time. To neither of these two experimental factors can the conditions here described be attributed.

It is quite possible and indeed the writer inclines to this opinion, that the treatment with X-rays serves to emphasize the vesicular condition of the chromosomes to make more clear relationships which undoubtedly already existed. Miss Carothers finds that X-ray treatment tends to increase the ease with which she finds chromatin granules in the peculiar vesicular condition which she calls "chromosomal vesicles."[1] It is possible that the fact that I first studied the radiated material is accountable for the recognition of the conditions here, although having once worked out the facts there I had no trouble in verifying the observations in normal untreated and uncrossed *Fundulus* eggs. Many of the findings have been confirmed by Miss Pinney in a study of *Fundulus* eggs as yet unpublished, and I have demonstrated my slides to various workers at Woods Hole, none of whom had any difficulty in seeing the conditions here described.

This account offers a rather different history of chromosome behavior from that usually given. The transition stages from one step to another are very clear, however, and do not usually permit of any other interpretation, so far as the writer can see, than the one here set forth. The new account of chromosomal formation and processes connected therewith, it will be seen, is easily fitted into the orthodox interpretation without doing violence to any important conception of mitotic behavior. It is

[1] This use of the term is unfortunate in the light of its earlier application in the sense used throughout this paper. "Chromosomal vesicle" in the sense of Sutton and Conklin refers to a vesicular condition of the whole chromosome. Miss Carothers has subsequently pointed out that the structure she described is in reality a plasmosome vesicle. (Jour. of Morph., 28, p. 465, 1917.)

on the details of the transition from one phase of mitosis to another that the new conception obtained from the study of the processes in *Fundulus* throws light, and at the same time offers a "raison d'être" for the appearances seen.

Fundulus cleavage cells show nuclei which are favorable to the study of these processes for the very reason that they are small in relation to the large amount of cytoplasm present. Because of the physical conditions thus engendered, the chromosomes and chromatic structures have a characteristic loose arrangement which permits their close study. At the same time this renders a study of individual chromosomes, as has been done for instance in orthopteran material, impossible. The behavior of a group of chromosomes as a whole is easily followed, but individual differences as in size in metaphase chromosomes are so slight that it is quite beyond the range of practicability to follow a single chromosome through the various metamorphoses it undergoes.

The material used in this investigation consisted of *Fundulus* eggs, fertilized as already stated by either *Fundulus* or *Ctenolabrus* sperm. Eggs were preserved during the early stages of cleavage (first five cleavages), and also during the later stages of development, but the cells from the early stages are the most favorable for cytological study, and it is chiefly from them that the figures are drawn. Fixation was most satisfactory in Bouin, although other fluids were also used. Iron hæmatoxylin, variously counterstained, gave the best results. The blastodiscs were removed from the yolk and embedded and sectioned separately.

Observations.

The metaphase plate may serve as a convenient point at which to begin observations on the mitotic process. Fig. 1 shows the conditions of chromosome and spindle relation in the normal *Fundulus* eggs at the time of maximum condensation of the chromosomes in the metaphase. It is from a blastomere in the fifth cleavage division. The cytoplasmic mass of such a blastomere is quite large as contrasted with the volume occupied by the spindle, and of course is large in comparison with the amount of chromatin present. This figure shows conditions

similar to those which led Van der Stricht to conclude that the nuclear sap passes to the poles of the spindle and is equally divided by the ensuing halving of the blastomere. The fibers are apparently grouped into bundles leaving spaces along which the clear streams of nuclear sap may flow to the poles. Van der Stricht regards this as evidence that the sap of the dividing nucleus gives rise to that of the daughter of nuclei.

In general the events which take place during the metaphase of mitoses in these eggs at least during the earlier cleavage divisions, do not differ greatly from those occurring regularly in the majority of animal and plant cells. It is with those phases of the mitotic cycle that follow the metaphase and continue until the stage of formation of the new metaphase is reached, that are of especial note here.

Fig. 2 shows the conditions obtaining at the moment of chromosomal division. Three of a group of chromosomes are here half divided and the ends are pulling apart preparatory to their separation in the anaphase. Already it is possible to detect the chromomeres of which the chromosome is composed. The metaphase represents the stage of maximum contraction of the chromosomes. It seems that at the moment of separation the chromosome begins to loosen up. This may be interpreted to mean that their permeability increases at this time; at any rate they take in liquid (water or cell sap?) resulting in the swelling up of chromosomes and consequent separation of the chromomeres. It is in this manner that a chromosomal vesicle begins its formation. As a result of this process the chromosomes lose their property of staining densely, and until they have passed well into the telophase, the walls of the vesicles thus formed retain the chromatic stain longest. As the vesicle grows, however, it is seen that the latter characteristic is really misleading, for the walls are merely lined with very fine granules of chromatin which later become separated by the increasing growth of the vesicle.

Fig. 3 is an anaphase. The loosening up process here has brought out the true character of the chromosome. A chromosome consists of two substances, linin and chromatin, of which the former is in the nature of a sheath or sac, while the latter

exists as a mass contained in the sheath. Conklin has described a similar structure for *Crepidula* chromosomes which consist of "chromatin enclosed in a linin sheath." Figs. 4 to 7 show successive steps in the anaphase and the various steps in the process of vesicle formation. The chromomeres break up and the granules of chromatin become peripherally arranged, and the center is for a while free from granules.

This central cavity of the chromosome does not seem to be a definite vacuole, as is figured for instance in some plant chromosomes, but is simply the space within the walls filled in from the fluid portion of the cytoplasm. The vesicle is achromatic in its interior due to the superficial distribution of the chromatin. Gradually there appear very fine strands or fibrils, doubtless of linin, growing from the vesicle wall inward and the granules of chromatin which accompany them are particularly noticeable at the intersection of the fibrils. Later the fibrils become stronger and somewhat reticulated, while the granules on the inner wall are farther and farther apart, and the vesicle is itself in appearance a tiny nucleus in the typical resting stage. When the reconstruction of the nucleus is complete, the granules are small and fairly equally distributed and do not stain very densely. At no time, however, does the nucleus become completely achromatic as is held in some cases. The behavior of the chromosome during its reconstruction permits a very rough comparison to an elastic bag tightly packed tightly with a fine granular substance. As the bag takes in liquid it swells up and the non-soluble granular substance becomes rolled up into tiny balls on the inner surface, and occasionally chains of the granules extend into the interior.

This interpretation of the chromosome is not unlike Conklin's view that these bodies consist of chromatin enclosed in a linin sheath. As they move to the poles they are transformed into vesicles, the interior of which becomes achromatic, "though frequently containing a nucleolus-like body, while the wall remains chromatic. These vesicles continue to enlarge and then unite into a 'resting nucleus'; the nuclear membrane is composed of the outer walls of the vesicles, while the inner walls stretch through the nucleus as chromatic portions." The

formation of chromosomal vesicles in the anaphases of ova has not been a matter of infrequent observation; and this phenomenon is not commonly seen in cells other than egg cells. In other words, the formation of chromosomal vesicles is, without much doubt, correlated with the presence of an unusually large amount of cytoplasm. As the chromosomes are transformed into vesicles, they absorb, it seems, large amounts of the achromatic material from the cytoplasm, by which they grow to resemble the familiar reticular structure. Their true nature becomes masked, but as will appear later, is not really changed. Vesicular chromosomes have been described in fish eggs by Moenkhaus and certain of the figures of Miss Morris indicate that she also saw them.

According to the usual interpretation, and to this Moenkhaus subscribes, the individual vesicles fuse with their neighbors and these larger ones with each other until at last the entire nucleus is simply one great vesicle. Moenkhaus describes the transformation of the chromosomes into resting nuclei as taking place by their conversion into vesicles which during an early stage can be distinguished into two groups by the difference in size. Then the smaller vesicles "fuse at first into larger ones, giving rise to a lobed nucleus. At this state it is no longer possible to tell the two kinds of vesicles apart. The fusion continues until a single well rounded nucleus results, with all traces of its double character lost."

Increase in size of the chromosome continues through the telophase stages and to the resting stage. A rough calculation of the growth from the stage (Fig. 7) where the definite vesicular character of the chromosomes (late anaphases and early telophases) is first discernable to the full size found in resting nucleus (Fig. 12) indicates a fivefold increase. It is scarcely possible to compare quantitatively the amounts of chromatin in the condensed chromosome and in the disperse vesicular stage. But one cannot easily doubt that an actual increase has taken place in the latter case. It is during this time in all probability that the new chromatin is formed in preparation for the ensuing division; and at the same time growth in the amount of achromatic substance present would also take place. The swelling

up process must of necessity be reversed at the prophase to a certain extent, and some of the liquid which had been taken in be expelled; as will be described later some shrinkage does actually take place.

During the transformation as well as subsequent to it two kinds of vesicles can be distinguished on the basis of size in the D series, in the control, and in the A series. The larger ones represent the *Fundulus* chromosomes and the smaller ones those from *Ctenolabrus*. Since in these three sets of experiments the eggs and sperm were equally injured (or in the control, were equally uninjured) by the radiation, exactly that result was to be expected in keeping with the findings of Moenkhaus and Miss Morris. The B and C series have contributed little to this phase of the investigation, but the conditions, while more complex there, apparently offer confirmation of the fact discovered by Hertwig that chromatin from radiated nuclei does not take part in the spindle to any great extent after development is initiated; or, as they have described it, the development in a strict sense thus becomes parthenogenetic. It is not to be expected therefore that the sperm chromatin in the case of the radiated B series, nor the egg chromatin in the C series will make any considerable contribution to the development of these hybrids. Such condition is found to obtain at least so far as my study has extended.

Figs. 8 and 12 represent various nuclear changes during the telophases. The cytoplasmic constriction begins to cut into the cell at about the time of Fig. 8; Fig. 12 was drawn from a cell in which the division was practically complete. There is very little difference between the latter and the resting stage shown in the next figure. During the telophase, growth of the vesicle continues and the distribution of the chromatin granules takes place. Thus the vesicles become approximated to each other so that finally there remains no space between them and each has conformed to its neighbors in assuming its final shape. Yet at all times they can be seen to retain their distinctly individual character, and in well fixed material the outlines of each can be followed. The figures are inadequate to represent the conditions for they are drawn all at one level, and the advantage that

careful focusing gives in studying their structure is lost. Some of the vesicles are more distinct than others, however, as is shown in Fig. 10, where one of them stands out with especial clearness. It may not be objected to these figures that they are tangential sections and that the vesicles really fuse in the center of the nucleus, for many of these nuclei extend through three sections and in each of the sections the same conditions are seen; if this condition were due merely to the tangential cut of the section the second of a series of three would not show the vesicles separately as it actually does.

Reconstruction consists in the further swelling up and rounding out of the anaphase vesicles and their close approximation to each other in the telophase. In the completely reconstructed nucleus the approximation of the vesicles is very close, so that they are no longer to be recognized as separated bodies. Yet the appearances do not indicate any actual fusion. One sees a nucleus consisting of vesicles so closely applied to each other that they conform in shape each to the other, and there are left no spaces between them. The inner boundaries are less sharp than the nuclear membrane, but even the nuclei which are only lightly stained show areas marked off from each other which represent the tightly packed vesicles. In every resting nucleus, whether radiated or not, which I have studied these areas can be made out. Fig. 15 represents a fairly typical case, although in other nuclei the vescular structure is even more apparent. That these vesicular areas do not represent simply a delay in the complete fusion can be seen by referring to Fig. 22 where their walls are still distinct, although the new chromosomes for the next division have been formed.

The resting condition of the nuclear vesicles is shown in Figs. 13, 14 and 15. The first two of these are from eggs before the first cleavage division; until they were found the writer did not feel sure that the vesicular condition represents more than a delay in the fusion and that the final result might be such as Moenkhaus has described. Since in these cases there had been no preceding division, however, and since it does not seem that the unfused condition would persist so long as from the maturation division and over the fusion of the pronuclei if the parts do

not normally coalesce, these resting nuclei gave additional credence to the belief that the vesicles maintain themselves over the resting stage as separate entities. Subsequent observations on the prophase render this position indisputable. (See below in connection with Fig. 22.) Careful study under very high power with proper conditions of lens definition and illumination on well fixed material leave very little doubt that the vesicles do remain separate. It is true that a one-to-one correspondence cannot be established between the number of vesicles in the resting stage and the number of chromosomes in the metaphase plate. This is true for two reasons: it is not possible to count with accuracy the number of these chromosomes when they are most condensed and therefore have the sharpest outline; and it is even more impossible to count the number of vesicles, for the plane of the section usually passes through some of them, and it is too uncertain to attempt to superimpose the vesicles of one section on those of the other to make a count, while at the same time all of the vesicles cannot be seen in a preparation of the nucleus as a whole. The inability to count the number of vesicles, however, is hardly an argument against their permanence for the reason that they can be followed through the interkinesis and prophases as is shown in the later figures.

These observations on the method of the reconstruction of the nuclei have a certain bearing on the problem of the nature of the nuclear membrane. Here, at least in its origin, the membrane is formed of the outer walls of the chromosomal vesicles. It may well be that the nuclear membrane of the resting cell is more complex than this, due to the interaction between nucleus and cytoplasm; indeed, some hold that the outer part of the membrane is of cytoplasmic origin, and is therefore more of an inner limiting membrane to the cytoplasm than of a constituent part of nucleus. The writer sees evidence for this view on Figs. 24 and 25 where more or less of the nuclear membrane still remains, although the inner vesicular walls have broken down and the new chromosomes have already formed.

Nuclear membranes of the *Fundulus* type which are really the outer vesicular walls find a parallel in the membrane formation around single chromosomes which have become abnormally

isolated, and have grown into small nuclei distinct from the true nucleus of the cell. Such a case Boveri described for *Ascaris*. Except in regard to size and the number of chromosomes entering into them, these smaller nuclei have the same structure as those of regular types, and their membranes cannot be distinguished from that of the larger nucleus. Boveri's minute pathological nuclei are themselves an argument for the view that the chromosomes retain their independence in the normal completely fused nuclei which is usually described for this form. In these hybrid fish eggs, fundamentally the same condition obtains, for the vesicles, closely applied to each other as they are, actually retain their identity, although no cytoplasmic layer intervenes between them. Vedjdowsky has expressed a somewhat similar view in regard to the nuclear membrane for he holds that it represents the peripheral portion of the certain chromosomes that have become vacuolated in their interior.

Experimental vesiculation of chromosomes has not proven difficult to bring about in cleaving eggs. Years ago Boveri published the results on *Ascaris* eggs just mentioned, in which the chromosomes may fail to fuse and each form its own nucleus-like vesicle, and his observations have been repeatedly confirmed. Conklin's numerous experiments on *Crepidula* eggs ('12) will suffice to show how common a phenomena vesiculation is. By various means the chromosomes of the eggs were prevented from fusing and each formed in the resting stage an independent "karyomere" quite comparable to Boveri's cases. Other instances might be cited.

A phenomenon which may be so easily induced as this one is most readily understood if one thinks of the normal nucleus in interkinesis as consisting of closely crowded entities, the vesicles, derived from the telophase chromosomes, which never normally coalesce. This means that the unusual conditions of the experiment merely push them a little apart from each other. As in the case of a catalyzed reaction, a condition which already exists may be modified easily, while it is very difficult indeed to bring about an entirely new set of relationships between elements not normally reacting.

Conklin has developed the idea of karyomeres, or chromosomal vesicles, basing it upon his observations and experiments, to such a stage of completion that he can show all stages of combinations from single vesicles on up to that of the entire complex. He figures cases that very closely resemble amitosis as shown by various workers, and suggests that this superficial resemblance may be a source of error in some at least of the observations upon which the claims of the occurrence of amitosis have been made. This suggestion may be reiterated here, for it is obvious that if there should be an indention between two of the vesicles in the resting stage of a *Fundulus* nucleus, direct division would be at once called to mind.

The polarity of the cell is definitely maintained in dividing *Fundulus* eggs. As the chromosomes pass to the poles their long axes lie in the cell axis, which passes through spheres and nuclei, and the vesicles are formed with this polarity. Cytoplasmic division follows only slowly after nuclear division and the nucleus has entered upon the phase of interkinesis by the time that the cytoplasmic separation is accomplished. Also before cytoplasmic separation, the centrosome divides and each half traverses an arc of 90° in preparation for the next division. This results in the establishment of a new cell axis at the right angles to the old. Thus for a time the old and the new axes exist together, the daughter nuclei themselves marking the old and their divided centrosomes the new. Fig. 16 is such a case, the two cells shown having arisen from the result of the division of an ectodermal cell of a blastula. The line connecting the points *A* and *B* represents the axis of the parent cell, while *CD* and *EF* respectively mark the axes of the daughter cells. The fact that the two latter are nearly perpendicular to each other is of significance when it is remembered that in the next division the upper of these cells will produce the two ectodermal cells and the lower two mesodermal.

The polarity is recognizable in the vesicles in many cases, the longer axes of the vesicles in general lying in the cell axis. It is, therefore, evident that about the time of centrosomal separation the polarity of the vesicles changes. Earlier oriented with respect to the old sphere, they have now shifted to the newly

established axis. Whether this is by an actual revolution of the entire vesicle, or only by a change of shape so that its short diameter now becomes its long axis is not clear, but it seems probable that the former process takes place. When condensation occurs and the new prophase chromosomes are formed they show the polarity in a very striking manner, almost every chromosome in the spireme stage being oriented in the long axis of the forming spindle (see Fig. 22). This of course is to be expected in view of the definite polarity of the vesicles in the prophase as shown in Fig. 17.

In reconstructing nuclei where the fixation is not quite perfect, the walls of the vesicles sometimes cannot be seen. They are very delicate and require a very complete fixation if they are to be preserved in their entirety. When the nucleus enters upon the first steps of the prophase and liquid is extruded from the vesicles so that they shrink or condense, the walls are particularly difficult to preserve. This results in an artificial running together of the granules into irregular clumps. Yet the area formerly occupied by the vesicle is still to be seen separating one clump of chromatin from the next, and the nuclear membrane is still retained although in a shrunken condition. The chromatin so massed by the irregular plasmolysis of the walls of the vesicles in some instances comes to resemble a prophase chromosome. Mere clumps of chromatin granules, however, with nuclear membrane intact and the inner vesicle walls gone indicate artifacts rather than normal conditions. Yet even they serve to suggest certain facts which are represented in the normal nuclei.

The new chromosomes arise within the old vesicles. When the writer first began the study of this phase of the subject, he was led to expect that the shrinkage which takes place at the beginning of the prophase as shown in Figs. 13, 17, and 18, would continue and the formation of the new chromosomes would be simply the reverse of the process by which the vesicles had been formed. Such is not the case, however, for it is now certain that the new chromosomes arise endogenously. The formation begins within the vesicle with the gradual increase in size and the aggregation of the chromatin granules upon its walls. The typical condition of the vesicles during rest shows small

granules distributed fairly uniformly throughout it. Succeeding this stage of general chromatic dispersion the vesicles appear to shrink, expressing part of their water content; this phenomenon is manifested doubly, first by the clear non-fibrillar area about the nucleus, the extent of which before shrinkage is thus marked, and second by the concentration of the contents of the vesicle, which in fixed material appears denser and takes more orange G than before. Coincident with this change the amount of chromatin appears to increase and the aggregation of the granules takes place, the larger masses of chromatin appearing along the periphery and perhaps extending into the center along a single thread. The aggregation continues until masses the size of the chromomeres of the anaphase are formed and have become arranged in a linear series. The linin sheath about this linear aggregation of chromomeres forms early. This structure is the new chromosome and must be thought of as chromosomal in nature quite as much as the more familiar metaphase stages. When it is completely formed the walls of the vesicle may be seen to be gradually dissolved and the denser contents to diffuse into the cytoplasm. About this time or a little later the split of the new, or filial, chromosome takes place, each chromosome and chromomere showing it about equally.

Figs. 17 and 18 show the first sign of the next division. The chromatin granules are well dispersed and the vesicles definitely oriented, while the pre-mitotic shrinkage has taken place. In Fig. 19 the aggregation of the chromatin granules has begun and the vesicles in this state can always be recognized as about to divide. In Fig. 20 is seen the linear arrangement of the chromomeres, a process which is well advanced in Fig. 21. In this latter case indeed the chromosomes are well formed and the linin sheaths can be made out. In these figures the walls of the vesicles are not as clear as in some cases, but the denser gel-like portion still shows the form and relationships of the vesicles. There would seem to be some variation as to the exact time when the disappearance of the walls takes place, in relation to the development of the spindle structures at least.

Figs. 21 and 22 are prophases in the spireme stage, early and later. Fig. 22 is an especially clear case showing the newly

formed filial chromosomes while at the same time the walls of the old vesicles have not yet disappeared. In the upper part of this figure they are less distinct, but elsewhere the vesicles are easily seen in their entirety. The walls stand out clearly and take the orange stain strongly, so that there can be no mistake about their presence. The spheres are not shown in the plane of this section, but the spindle fibers indicate the polarity with most of the chromosomes oriented in the spindle axis.

Of the greatest importance is the evidence presented in Fig. 22. The presence in the vesicles, the walls of which are still intact, of the new filial chromosomes, for these spireme structures are now definite chromosomes, is the crux of the argument for chromosomal continuity. It is not difficult to follow through the history of the vesicles from the time of their earliest formation out of the metaphase chromosomes thorugh anaphases, telophases, and interkinesis, and to see that the vesicle represents, so far as the chromosomal bodies are concerned, a continuous unit of structure. The parental chromosomes are not lost in the vesicle stage; they are merely metamorphosed, appearing in a new form and the chain of the continuity is never broken. In the vesicles the continuity is still maintained throughout the resting period of the nucleus and it is only in the late prophase of the daughter generation that the vesicular walls disappear. But before that disappearance, the new filial chromosomes have been formed and are in the so-called spireme stage, representing uninterruptedly the persistent continuity of the units with which the cycle started in the parental metaphase. In this spireme stage the chromosomes are as definitely chromosomes as in the metaphase which immediately succeeds, and they have only to condense further and to attach themselves to the spindle to enter the metaphase, a process obviously favored by the dissolution of the nuclear walls.

In Fig. 23 all traces of the vesicle walls have disappeared and the chromosomes resemble the late prophase chromosomes of many animals. This is the only case in which the longitudinal split is seen, and it may be that it is really precocious, or that the figure represents the facts only in part. In most nuclei in which the chromosomes are well formed the spindle development

has progressed farther than in this case. These split chromosomes are suggestive, however, when compared with such chromosomes as shown in Fig. 2.

The manner of attachment of the fiber to the chromosomes and the steps leading to the metaphase plate condition are shown in Figs. 24 and 25. Fig. 24 is an oblique section in which there is only one sphere. The walls of the vesicles at the polar end of the nucleus are gradually disappearing and the fibers are making their way to prophase chromosomes; traces of the walls however can yet be seen. In the next figure the attachment of the fibers is practically completed and the chromosomes have become definitely placed upon the spindle. While in general they were orientated in the prophase with their long axis in the spindle axis, they become definitely "drawn" into place with the attachment to the fibers. The condensation of the chromosomes and their final arrangement on the spindle completes the cycle and brings us again to the stage shown in Fig. 1.

These last two figures are interesting also for their likeness to the conditions found by Van der Stricht in the egg of *Thysanozoön*. In this form the spindle and its fibers can be differentiated into several parts. The principle cone, "cone principaux," of fibers corresponds to the mantle fibers and is composed of those which attach to the chromosomes themselves. Just outside these but still in the area of the nucleus is a cone of fibers which do not attach; this Van der Stricht called "cone accessoire." Finally out in the cytoplasm is to be seen a series of the fibers which interlock with those from the other end of the spindle; these are the "fibers cytoplasmique." In *Fundulus* eggs exactly the same conditions seem to prevail (Figs. 25 and 1).

As the central spindle fibers make their way to the chromosomes and attach to them there is a gradual movement of the chromosomes to the equator of the spindle to form a plate. During this movement the last stages in the condensation of the chromosomes takes place, resulting in the formation of the densely staining metaphase rods, such as were figured by Moenkhaus and Miss Morris. As they condense the chromosomes increase in thickness, so that the metaphase chromosome is both shorter and broader than that of any other stage. The nuclear

sap also becomes completely diffused into the cytoplasm by this time, and no distinction can be seen. The final event leading up to the formation of the plate is a change in position of the chromosomes with the reference to the axis of the spindle. Previously oriented with their axes parallel to that of the spindle, they now come to lie at right angles with their free ends pointing towards the periphery. When this stage has been reached the plate is fully formed.

Thus it is possible to follow out the history of the chromosome group in the early cleavages of *Fundulus* without a single break in the continuity from the metaphase of one generation to that of the next. It should perhaps be pointed out again that this account refers to no particular mitosis. In all of the early cleavage divisions in which the nuclear structures are large enough for study the facts are as here related.

Since making the above observations on *Fundulus* the writer has been able to extend them to many stages of the eggs of *Corregonus albus*, and to satisfy himself that the behavior of nuclear structures is essentially similar in both fish.

Figs. 26 to 29 do not add any direct proof to the theory of the continuity of the chromosomes, but they serve to throw a side light on the process of spindle formation, at least as it occurs in the gastrulæ of this form. The writer has not seen the same conditions in the early cleavage cells and does not presume to claim that they occur there, but since the gastrula shows these rather unusual phenomena, they may be set forth here to assist perhaps in the interpretation of similar appearances elsewhere. At the beginning of the prophase, the formation of the central spindle takes place between the centers that are here divided and are passing to the opposite side of the nucleus. This process, it will be noted, occurs at a relatively later period in the division of the gastrula cells than in the earlier cleavage cells. Fig. 27 is a face view of a spindle of this type a little farther developed; certain of the chromosomal vesicles become separated to make way for the developing spindle which gradually sinks down between them and is thus ready for the attachment of the fibers to the chromosomes whenever the latter are formed. It might be thought by analogy with such forms as *Crepidula* that

Fig. 27 really represents the male and female halves of the nucleus. That this is not the case, however, is shown by Fig. 28 in which only the *Fundulus* chromosomes are visible. Miss Morris found that in *Fundulus* cytoplasm the *Ctenolabrus* chromosomes are small round bodies, distinctly differentiated from those of *Fundulus*. This observation I can corroborate; the cytoplasm modifies the character of the foreign chromosomes, and they can be recognized as different from those of the egg. The egg from which these four figures were drawn was fertilized with *Ctenolabrus* sperm that had been subjected to X-radiation for fifteen minutes; this is sufficient duration of time for the radiation to kill the male chromatin, which therefore made no contribution to the development of the egg and is not represented, as is shown by Fig. 28 which contains only *Fundulus* chromosomes. Since there is no male contribution to this nucleus, the interpretation that Fig. 27 shows merely the male and female halves of the nucleus is not valid. Rather the true state of affairs is made clear by Fig. 29 which is a cross-section of such a nucleus as is shown in Fig. 27; the central spindle makes its way through the groove shown on the upper side of the nucleus.

Discussion.

(*a*) *Chromosomal Vesicles.*

While there are numerous cases cited in the literature which would seem to give the conception of the constitution of chromosomes and resting nuclei here set forth a fairly wide application, the writer does not care to attempt a generalization from these observations extending the principle to other forms, although some of the cases which support these findings must be given. Any generalization at this time would seem unwarranted for too many instances are known where neither the chromosomes nor the chromatin during rest seem to answer exactly to these descriptions. There are many cases, for example, in which the chromosomes, judging from their behavior in the prophases, may be regarded as made up in chromatin granules strung upon a linin thread, and it is even possible to identify the granules in homologous chromosomes. How such a structure is to be derived from a permanent vesicular chromosome is not yet clear, al-

though this derivation is perhaps thinkable. The account here given aims rather merely to present the conditions as they are found in the fish eggs which I have studied, and to point out that these facts may have significance for current cytological and genetic speculation.

Conklin in his studies on *Crepidula* (1902) has perhaps followed out the history of the chromosomal vesicles, or karyomeres, as he calls them, more completely than any other observer. Of the behavior of the *Crepidula* chromosomes he says: "In large cells where the divisions succeed one another at short intervals the chromosomes begin the growth characteristic of the daughter nuclei, the absorption of substances from the cell body, before they have fused together, whereas in small cells or cells which divide only at long intervals the chromosomes fuse before the absorption of achromatic material begins."

"The history of the nuclear changes during the cycle of division may be summarized as follows: (1) The chromosomes, consisting of chromatin enclosed in a linin sheath, divide and move to the poles of the spindle where they partly surround the spheres. (2) Here they become vesicular, the interior of the vesicle becoming achromatic, though frequently containing a nucleolus like body, while the walls remain chromatic. (3) The vesicles continue to enlarge and then unite into the 'resting nucleus'; the nuclear membrane is composed of the outermost walls of the vesicles, while the inner walls stretch through the nucleus as chromatic partitions; the chromosomal vesicles from the egg and sperm nuclei remain distinct longer than those from the same nucleus. (4) The chromatin of the inner alveolar walls then aggregates into threads, giving rise to a 'chromatic reticulum,' though the linin still preserves, for a time at least, the alveolar structure. (5) The chromatin of these threads then separates into spherules, which are connected together by linin threads; these spherules vary in size, and at first are solid, and stain alike. (6) They become hollow and are differentiated into oxy- and basichromatin. (7) In the first maturation, each of the basichromatin spherules, or bodies, grows into an individual chromosome; in the cleavage, the basichromatin spherules unite into several linear series, thus forming a segmented spireme.

(8) The oxychromatin spherules grow smaller and are dissolved in the nuclear sap while others are arranged in series on the linin threads into which they are formed; these threads with attached spherules form the spindle fibers." Thus the relation of the old vesicle to the new chromosome is followed out.

Evidently from this description there is a close parallel between the behavior of the chromosomes of *Crepidula* and those of *Fundulus* eggs. Much of this summary will fit, word for word, the conditions which have been delineated in the preceding pages, although in *Fundulus* the vesicles do not fuse in the resting stage.

Conklin points out the fact that "such vesicles are found generally, if not uniformly, in the early divisions of the ova, though they are not usually found in other mitoses." He attributes this to the difference in size and rapidity of division of the blastomeres as compared with tissue cells, and concludes "that in the large cells where divisions succeed each other at short intervals, the chromosomes begin the growth characteristic of the daughter nuclei, *i. e.*, the absorption of substances from the cell body, before they have fused together, whereas in small cells or cells which divide only at long intervals the chromosomes fuse before the absorption of achromatic material begins." With this conclusion the facts as found in *Fundulus* in general are in accord.

In both kinds of eggs also the growth stages of the daughter nuclei are quite alike. In *Crepidula* "after the fusion of the chromosomal vesicles to form the daughter nuclei, the latter continue to absorb achromatic material, growing larger and larger until the prophase of the next division. A part at least of the achromatic material absorbed is derived from the sphere which in turn contains interfilar substance of the spindle and aster. This recalls the conclusions of O. Hertwig in which he points out that in the formation of the daughter nucleus the chromosomes absorb 'Kernsaft' and become vesicular, the process being the reverse of what occurs in the beginning of division where 'Kernsaft' is set free into the cell body. A similar view was held by Butschli . . . ."

The most important point of difference between Conklin's

observations and mine is the matter of ultimate fusion of the chromosomal vesicles in the resting stage, and the formation of the new chromosome in the vesicle. In *Fundulus* there can be no doubt that a direct continuity exists between vesicle and prophase chromosome and that the vesicles do not fuse during the rest period. One is compelled to suspect that *Crepidula* offers less favorable opportunity for the study of this particular detail than *Fundulus*. Conklin himself believed that there is a real independence of the vesicles although they appeared to fuse, and it may be inferred from the following paragraph that he expected their persistent identity throughout the resting nucleus to be proven in time. The observations on *Fundulus* may be taken as a confirmation of his view expressed in 1902.

"It sometimes happens, especially in eggs in which more than the normal number of centrosomes and asters are present, that some or all of the chromosomal vesicles do not fuse but remain distinct through the whole resting period. In such cases each of the vesicles behaves like a minature nucleus, absorbing the achromatic material and forming a network of chromatin either within the vesicle or on its walls. In this growth and differentiation the vesicle keeps pace, step by step, with the normal nucleus, so that one must regard the resting nucleus as virtually composed of vesicles, though their union may be so intimate as to hide this structure. The resting nucleus is not, therefore, a single structure any more than is the nuclear plate. It is composed of units each of which so far as known, has the properties of the entire nucleus, and the union of these vesicles into a single one may be considered as a secondary character. It is altogether probable that the chromosomes and hence the chromosomal vesicles, preserve their identity throughout the resting nucleus."

Moenkhaus also was unable to see in the nuclei of the resting cells evidences of separate chromosomes, yet he too, believed that the substance of each chromosome forms a persisting unit. We may expect then that the next step in the proof of chromosome continuity and persistence is the recognition of structures in the resting nuclei which can be homologized with the chromosomes of the metaphase plate. This I believe is accomplished in the fish eggs of my experiments. Moenkhaus states the case as

follows: "The question whether the individual chromosomes persist through the resting stage so that upon the resolution of the reticulum into the chromosomes the same component chromatin granules again go together to make the same chromosomes from which they were derived is a question first raised by Rabl and later definitely stated by Boveri. Since that time so much evidence has accumulated to support this conclusion that it has come to be rather generally accepted. Even a general review of the evidence is unnecessary here. Such a review would show that the fact has never been definitely demonstrated. Some of the most direct evidences yet given are the observations of Herla and Zoja on the *Ascaris* hybrids in which it was shown that the small chromosomes of the variety *univalens* which entered the resting nucleus with the larger ones of the variety *bivalens* again emerged in characteristic form. Equally strong evidence is now afforded by my observations on hybrid fishes. Here, as in the *Ascaris* hybrid, two kinds of chromosomes enter the resting nucleus from which each kind again emerges. As long as the two kinds remain grouped, as during the two divisions, this fact has little added significance, since within each group it would be perfectly possible for the component chromosomes to exchange chromatin granules during the resting period. If, however, as occurs in the later cleavages, the two kinds of chromosomes become mingled the chromatin granules of both kinds must lie mingled together within the resting nucleus. If from a nucleus the two kinds again emerge, it amounts almost to a demonstration that the chromatin substance of a given chromosome forms a unit and that the unit persists."

The evidence presented by Conklin and by Moenkhaus most nearly touches the subject of my investigation of any that I have found in the literature. Both of these workers failed to see in their material that the chromosomes may actually be discerned in the nuclei during rest as distinct vesicles. Yet both believed that a continuity of structure does exist and that the chromosomes form persistent, independent units. My material offers more complete and direct evidence that the vesicles persist and leaves little room for doubt that the conclusion of the observers mentioned is the correct one.

Sutton (1900) was the first of a group of cytologists working on the chromosomes of grasshoppers to point out that in the early stages of nuclear formation each chromosome forms a vesicle about itself, but thought that later the proximal ends of these fused, a condition which he interpreted as supporting the idea of chromosome individuality. His conception of a chromosome is not unlike that of Conklin, and has since been amply confirmed.

The occurrence of the chromosomal vesicles is quite common in the eggs of animals, and it has also been reported in certain other tissues as well. The following list is presented merely to give a few examples; it is in no way complete, but will serve to show the wide distribution of the phenomenon.

Name, W. G. Von, Trans. Conn. Acad., Vol. X., 1899.	*Planorbis*, early development of eggs.	Figures vesicles in anaphases.
Goldschmidt, R., Zeit. f. wiss. Zool., Bd. 71, 1902.	*Polystomum* eggs.	Karyomerites, at first one to a chromosome; later fuse and are not recognizable.
Von Kemnitz, G. A., Arch. f. Zellf., Bd. 10, 1913.	*Branchycœlium* eggs.	Karyomerites which later fuse.
Grille, K., Arch. f. Zellf., 12, 1914.	*Gyrodactylus elegans* eggs.	Karyomerites which fuse later.
Boveri, Th., Merkel u. Bonnet's Ergebnisse, 1891.	*Ascaris* eggs.	Accidentally separated chromosomes produce vesicles, from which new chromosome arises.
Lillie, F. R., Jour. Exp. Zool., 12, 1912.	*Nereis* eggs.	Figures vesicles in telophase; later fuse in reconstruction.
Mead, A. D., Jour. of Morph., 13, 1897.	*Chætopterus* eggs.	Figures show what are probably chromosomal vesicles.
Lefevre, G., Jour. Exp. Zool., 4, 1907.	*Thalassema* first cleavage.	Vesicles in anaphases; later they fuse.
Wilson, E. B., The Cell, 1900.	*Toxopneustes* egg cleavage.	Figures vesicles in telophase.
Boveri, Th., Zellstudien, IV., 1900.	*Echinus* eggs.	Figures vesicles in anaphase.
Buchner, P., Arch. f. Zellf., 6, 1911.	Sea urchin.	Karyomerites in first parthenogenetic maturation division.
Konopacki, M., Arch. f. Zellf., 1911.	Sea urchin.	Vesicles produced experimentally; thinks of them as nuclear budding.
Schaxel, J., Arch. f. Mikr. Anat., Bd. 76, 1911.	*Strongylocentrotus* egg.	Vesicles in telophase; they fuse, then alveolize, producing fine nuclear network.
Bury, J., Arch. f. Entw., 36, 1913.	Echinoid egg cleavage.	Karyomerites in early stages.

Smallwood, M., Morph. Jahrb., 33, 1905.	Nudibranch eggs.	Telophase vesicles which later fuse.
Lillie, F. R., Jour. Morph., 17, 1901.	*Unio* eggs.	Figure of resting nucleus yet in reconstructing process shows vesicles.
Conklin, E. G., Jour. Phila. Acad. Nat. Sci., 15, 1912.	*Crepidula* eggs.	Experimentally produced karyomeres from single chromosomes.
Häcker, V., Arch. f. Mikr. Anat., 46, 1895.	*Cyclops* eggs.	A few small vesicles which are male and female contributions.
Ruckert, J., Arch. f. Mikr. Anat., 45, 1895.	*Cyclops* eggs.	Chromosomal vesicles in both male and female halves, in transition to resting stage.
Kühn, A., Arch. f. Zellf., 1, 1908.	*Daphina* parthenogenetic eggs.	Vesicles in telophase, but they do not prove continuity for they are unrecognizable during rest.
Amma, K., Arch. f. Zellf., 6, 1911.	*Cyclops* eggs.	Figures vesicles in four-cell stage.
Bigelow, M. A., Bul. Mus. Comp. Zool., XL., 1902.	*Lepas* eggs.	Figures show vesicles in early cleavage stages.
Sutton, S. S., Kans. Univ. Quart., 9, 1900.	*Brachystola* spermatogenesis.	Chromosomal vesicles which became fused at one end.
Otte, H., Zool. Jahrb., 24, 1907.	*Locusta* spermatogenesis.	Chromosomal vesicles which were thought to remain distinct.
Davis, H. S., Bul. Mus. Comp. Zool., 53, 1908.	*Locustidæ* and *Acrididæ*, spermatogenesis.	Vesicles fuse at one end.
Pinney, E., Kans. Univ. Sci. Bul., 4, 1908.	*Phrynotettix* spermatogenesis.	Vesicles which were thought to remain distinct through resting stage.
Wilson, E. B., Jour. Exp. Zool., 13, 1912.	*Oncopeltus* spermatogenesis.	No rest between maturation divisions; chromosomes crowd together, like vesicles, without loosening up; never fuse.
Wenrich, D. H., Bul. Mus. Comp. Zool., 60, 1916.	*Phrynotettix* spermatogenesis.	Vesicles retain their own limits in the nucleus in rest.
Medes, G., BIOL. BULL., 9, 1905.	*Scutigera forceps* spermatogenesis.	Vesicles in second spermatocyte; they fuse; but traces are often long retained.
Conklin, E. G., Jour. Phila. Acad. Nat. Sci., 13, 1905.	*Cynthia* eggs.	Vesicles in cleavages.
Moenkhaus, W. J., Amer. Jour. Anat., 3, 1904.	Eggs of *Fundulus* and *Menidia* crosses.	Vesicles which fuse, but believed to be distinct though invisible as such in rest.
Morris, M., Jour. Exp. Zool., 16, 1914.	*Fundulus* and *Ctenolabrus* crosses.	Vesicles which fuse.

Hertwig, G. & P., Arch. f. Mikr. Anat., 84, 1914.	*Gobius* and *Crenilabrus* crosses.	Vesicles in telophases which are thought to be signs of degeneration.
Van der Stricht, O., Arch. de Biol., 12, 1892.	*Triton* eggs.	Vesicles formed by looping of chromosomes; figures of resting nuclei suggest traces of vesicles.

(*b*) *Chromosomal Continuity.*

Chromosomal individuality has been so often argued of late years that a renewal of the discussion seems an almost trite and needless repetition. However, in the light of the facts here brought forth it may not be out of place to review the present status of the question. It must be understood that no argument is made that chromosomes are directly transmitted from one cell generation to the next as *identities;* that conception has long since been abandoned for it has been shown to be inadequate. Rather the argument is that the new chromosomes arise from the same structural substance as the old, a theory of genetic continuity. One prophase chromosome and only one arises from the substance of a single one from the preceding division. It is such a theory which is the subject of this discussion.

The hypothesis of chromosome individuality dates back to Rabl ('85) who "concluded that the chromosomes do not lose their individuality at the time of division, but persist in the chromatic reticulum of the nucleus" (Wilson). He thought that the disappearance of the chromosome in the reticulum was only apparent, for he could recognize in the reticulum of the salamander nuclei portions corresponding to the chromosomes. The reticulum is formed by the outgrowth of processes, secondary and tertiary, from the chromosomes which while fusing on the neighboring chromosomes, nevertheless, maintain their own identity.

That Rabl's view regarding the persistence of the chromosomes has not found general confirmation is indicated by the following quotation from Wilson, who, in discussing the theory of chromosome individuality[1] remarks "that in vast majority of cases the identity of the chromosome is wholly lost in the resting nucleus and the attempts to identify them through the polarity or other morphological features of the nuclear network have,

[1] The Cell, p. 300.

on the whole, been futile. It is, therefore, an abuse of language to speak of a persistent individuality of the chromosome."

There is, however, a great mass of evidence in favor of a view of chromosome individuality or rather, since there is so little evidence of actual chromosome persistence as unchanging and identical bodies during the resting stage, the hypothesis of genetic continuity of chromosomes. The fact that almost invariably the same number of chromosomes emerges from the resting nucleus that went into it, Boveri's studies of abnormal variations in the early development of *Ascaris* eggs, Zur Strassen's observations of the giant embryos of *Ascaris*, the previously mentioned discoveries of the independence of maternal and paternal chromatin in hybrid eggs, the constant recurrence of chromosomes in the same form and even position, *e. g.*, in root tip cells of *Yucca* (Clemens Müller) and in *Ascaris* eggs (Boveri), their gradation in size invariably repeated in certain insect germ cells, the history now well known of the accessory chromosome, which retains its identity from one cell generation to the next, the recent studies which have shown clearly the homologies of chromosomes other than the accessory as well as their independent behavior, and finally the correlation of the results of recent studies in genetics with such a behavior on the part of the chromosomes, all these points constitute strong inferential evidence in favor of a theory of chromosome continuity.

Chromosome continuity is a theory as applied to resting nuclei only. In the mitotic stages the facts are clear; the chromosomes are distinct bodies which behave as units and it is a general observation that those of one cell are homologous with those of others in the same organs and in general in the same species. It is only in the interkinesis that the facts partake in any way of the nature of a hypothesis and admit of differences of interpretation regarding the point here discussed. In this state it is difficult or impossible to recognize chromosomes as distinct entities and accounts which seek to distinguish them throughout rest have been and must be received with great caution. Adverse criticisms of the theory have been based upon claims of faulty technique, faulty observation, upon obvious objections to a hypothesis of strict individuality (which is impossible both in a

chemical sense where two phases exist in the same cell, and where there must be interaction between the constituent parts of the system, and in a mathematical sense of unchangeableness) and to the more deeply grounded objection that the process is one of dynamic character and is not subject to morphological explanation.

Mathew's objection is perhaps the most significant of the first group. He says "All so-called nuclear stains of basic nature except the mordanted stains as iron hæmatoxylin combine with nucleic acid. Cytologists in following chromatin and chromosomes may be following only the inert skeletal material of the nucleus while the active albuminous material is entirely neglected since it does not gel or stain with basic dyes." Yet significance must attach to the *constant recurrence* of these structures whether they are of skeletal nature or otherwise, and to the fact that chromosome structures reproduce themselves and have done so indefinitely. If they are not more than skeletal in nature, they are at least the manifestations of the "protein or basic" and perhaps more active parts of the cell, and the correlation is so close that their behavior is no less significant.

Fick's objections as Wilson has pointed out refer only to the strict interpretation of individuality which is not supported by the evidence and which is no longer held by cytologists in general.

With regard to the morphological method of attack as one means of studying dynamic problems more is to be said in defense than against. The nucleus is, indeed, "a dynamic system," as one of the chief critics of the continuity hypothesis has pointed out; and the process by which it reproduces itself is of all biological phenomena the best example of a dynamic process. Yet a dynamic process whose basis is not material, indeed not morphological, is only with difficulty thinkable. In whatever may be the final terms of our thinking, chromosomes, chromomeres, molecules or ions, we deal in the end with structure and configuration; and this fact must make us hesitate in any attempt to detract from the significance of chromosome structure and continuity. We may believe consistently that the chromosome is a continuing structure, and that its correlations with processes of heredity are even so close as to be causal, without forgetting that

its functions and behavior are dynamic. Child has pointed out with great propriety that "instead of being the basis the chromosome is itself a problem of heredity." We no longer think of chromosomes as ultimate units of structure or the unreducible cause of heredity processes. It is true that the heredity processes have been merely pushed farther back into the cell for explanation; but even then, a distinct step has been taken if the correlation can be traced as in *Drosophila*, between body characters and chromosomes; and a more complete dynamic explanation of the processes, along with that of chromosome structure and behavior, can only be eagerly awaited. Dynamic processes are of course at the basis of all biological functions; with the physical structure of the organic body they do their work, and new facts, if real facts, must be comprehended in the larger dynamic system. Biology is now well launched out in the attempt to find dynamic solutions to its problems, and morphology including cell morphology still has contributions to make to the attempt. It is difficult indeed to understand the structure of a mechanism without some knowledge of its function, but it is equally difficult to appreciate a structureless function.

Of interest from the standpoint of chromosomal independence are the observations now being carried on by Chambers on the micro-dissection of the living cell. It appears from this work that there is gradually being established from the living material a confirmation of the general principles of cell structure and behavior as worked out on fixed preparations. The fact that certain structures as polar radiations, are not visible in the living cell and are present in fixed cells, perhaps due to injury or precipitation caused by the fixing process, is not an argument against their significance since they occur constantly and in precise relations to other structures. If not significant in themselves they are at least manifestations of processes which lie more deeply in the cell. Of particular interest here is the observation as yet unpublished of Dr. Chambers, of which he kindly permits mention to be made here, that in grasshopper spermatocytes the telophasic chromosomes are to be found in the vesicular condition. When single grasshopper chromosomes are removed and allowed to grow in plasma they develop into round

vesicles which by puncturing with the needle can be made to collapse.

From the work of many cytologists there has gradually accumulated a great mass of inferential evidence for chromosomal continuity which is well known, and the general nature of which was mentioned earlier in this paper. The form and size relations of chromosomes, the behavior of the sex chromosomes, which can be followed from one generation to the next, the homologous character of somatic chromosomes, certain chromosomes not correlated with sex (according to the work reported by a number of investigators, *e. g.*, by Wilson and by Carothers), all of these facts can be understood only with the greatest difficulty on any other basis than that of continuity of structure. A very recent case is Wenrich's finding that in *Phrynotettix* there exists a pair, "B," whose architecture is constant for all individuals studied, and another pair, "A," which is recognizable "through all stages from spermatogonia to spermatids." This case is very clear and of great importance.

Vejdovsky's ingenious theory is of interest in relation to my observations, although I do not find that the chromosome behavior of *Fundulus* tallies with his descriptions of observations upon which he bases his theory. He holds that the old chromosomes produce anlagen from which the new ones arise. As each organic individual comes from the anlage in the female organs, so do the chromosomes also arise from anlagen. The old chromosomes produce only anlagen for the new generation and the formation of the latter sets in with the beginning of the new cell. This anlage is the chromonema which gives rise to the two substances of which chromosomes consist, chromatin and linin. Each chromosome begins its existence with a spiral chromatin thread while the linin substrate of the old thread is lost to the anlage of the "Kernenchylem."

Reference must be made to the bearing of genetical work on the doctrine of the significance of chromosomes. The results of the great mass of recent work in this field demands as a practical necessity that chromosomes be treated as if they are actually persistent individuals, and the genetical behavior of heredity characters is being steadily brought into line with chromosomal

behavior as a more thorough-going knowledge of the latter is obtained. It is a noteworthy fact that those cases where heredity does not seem to conform to the expectation based upon the behavior of chromosomes are usually cases where the chromosomes are small, numerous and imperfectly known, and that in general the difficulties in comparing the two sets of facts have to a large extent disappeared with increasing knowledge.

The significance of hybridization experiments for the doctrine of chromosome continuity has been repeatedly pointed out, and a single case will serve to illustrate it. Federley crosses different species of the moth *Pygæra* in which the diploid and haploid chromosome numbers are as follows: *P. anachoreta*, sixty and thirty; *P. curtula*, fifty-eight and twenty-nine; and *P. pigra*, forty-six and twenty-three. In whatever cross was made of these three species the chromosome number of the hybrid was the sum of the two haploid sets that went in. According to Federley the chromosomes preserve their continuity through many generations of foreign cytoplasm.

Evidence that there is chromosome continuity in plants is quite abundant, as may be seen for example, from the reviews of Stout and of Wenrich. Only the main trends of the evidence need be mentioned here. First of these are the findings of Rosenberg, Overton, Lundagård and others that in the somatic cells of a considerable number of plants there occur in the resting nuclei chromatic bodies of the same number as the chromosomes during metaphase: these bodies enlarge directly into the prophase chromosomes. To certain other types of plant nuclei the views of Gregoire and his school apply. According to this idea the chromosomes as they pass to the poles become vacuolated, or alveolized, often forming in the resting nuclei reticulate bands which later are transformed into a spiral thread, the prophase chromosome of the next division. Of especial interest because of the very suggestive similarity of the method by which chromosomes are formed in *Fundulus*, is the view of Bonnevie resulting from the study of both plant and animal cells, *Allium* and *Ascaris*. She believes that there is genetic continuity of chromosomes although there is no *identity* between those of different mitoses: from the substance of each chromosome at the end of its life

however, there arises endogenously the new prophase chromosome. The old chromosome is an earlier existing "endogen" for the foundation of the new. She regards the chromatic substance as the persistent continuing portion, while the achromatic substances between cell generations are lost. My observations on *Fundulus* bear out the main facts of this conclusion, although the details of the process differ.

It remains finally to inquire whether any significance attaches to the fact that the conditions described in this paper occur in tissues that are dividing rapidly. In all cases where persistent chromosomal structures are found the period of interkinesis is comparatively short; the question may be raised therefore whether in cells in which the interkinesis is of long duration the fusion of the vesicles is not more complete and the continuity perhaps lost. It seems to the writer that this is probably not the case, although the latter cells may require a more delicate technique and more close observation to discover that the vesicles are persistent entities. The fact that there is no resting period intervening between the maturation divisions and that the chromosomes in this case persist from one mitosis to the next in itself argues for the view of continuity elsewhere. We may look upon this discrete nature of these chromosomes as a manifestation of a condition that is general for cells and is merely emphasized in this particular case. A somewhat similar principle is that of a catalyzer, which is not able to initiate a reaction, but merely to hasten one that ordinarily takes place at a much slower rate. A second reply to the question of the significance of the above mentioned fact is that usually cells which divide very slowly are not of hereditary importance since those cells which bear the hereditary qualities usually divide with fair rapidity.

Summary.

The chromosomes in the eggs of *Fundulus* can be traced from the metaphase of one cell generation through all the stages of mitosis and interkinesis as continuous structures which give rise to the prophase chromosomes of the next mitosis. As they pass toward the poles in the anaphase they gradually loosen up, showing their constituent chromomeres, and finally form vesicles

which come together in the telophase. Although they are closely applied to each other in the resting nucleus, they still maintain their unity of structure. When the nucleus begins the next division the new chromosomes are produced endogenously, each within the substance of one of the old vesicles which persists until the new chromosome is well formed. Thus there is clearly a continuity of substance from the old to the new metaphase chromosome. These observations establish clearly the genetic continuity of the chromosomes for this case and the evidence in general is so strong that the fact may be held as of wide application. The preceding account gives in detail the facts as observed in the mitoses of *Fundulus* eggs, a general summary of the occurrence of chromosomal vesicles and the relation of other cases to the present one, and finally a general statement of the present status of the theory of genetic continuity of chromosomes with the main points of evidence for it.

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EXPLANATION OF PLATE I.

These figures were all drawn with the aid of a camera lucida and Leitz two mm. objective with twelve compensating ocular (except figure sixteen as noted below). They are reproduced here at a magnification of a little more than eighteen hundred diameters.

FIG. 1. F♀ × F♂ normal; fifth cleavage division. Metaphase plate of which the chromosomes of two levels only are shown. Stage of maximum condensation of chromosomes.

FIG. 2. F♀ × C♂. Both radiated for three minutes before fertilization; second cleavage division. The beginning of chromosome division and first signs of separation of chromosomes are shown in this part of metaphase.

FIG. 3. F♀ × C♂; eggs radiated for fifteen minutes before fertilization; second cleavage division anaphase. The loosely formed chromosomes show increased size, the linin membrane and the chromosomes.

FIG. 4. F♂ × C♂; sperm radiated for fifteen minutes before fertilization; second cleavage division; chromosomes in similar condition to Fig. 3.

FIG. 5. F♀ × C♂; sperm radiated for three minutes before fertilization. The beginning of the vesiculation of the chromosomes. Liquid is being taken into the linin sac which in consequence swells up. A few of the chromosomes from both ends of the anaphase spindle are shown.

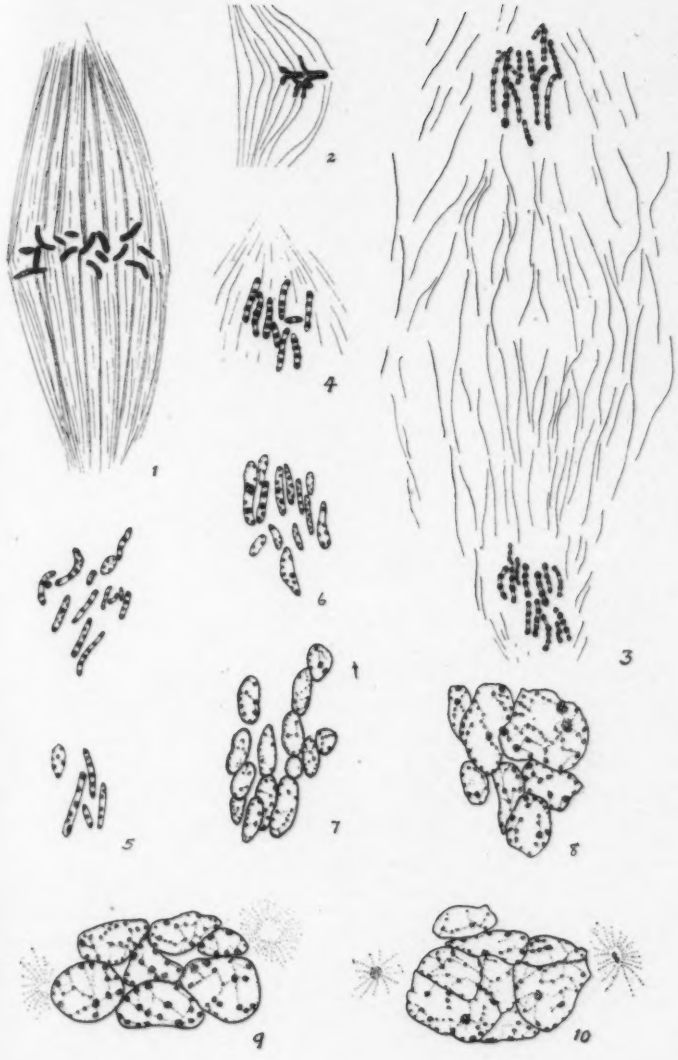
FIG. 6. F♀ × C♂; eggs radiated fifteen minutes; from one pole of anaphase of second cleavage. Same process carried farther.

FIG. 7. F♀ × C♂; both radiated three minutes; third cleavage division. A section through one pole of anaphase. All chromosomes are vesicles, showing no fusion, no condensation. The swelling process is still proceeding. The arrow points to the position of the sphere.

FIG. 8. F♀ × C♂; both radiated three minutes; fifth cleavage division, telophase. A somewhat oblique polar view. Nuclear division completed, but cytoplasmic not yet fully accomplished. Chromosomal vesicles further swollen, but quite distinct.

FIG. 9. Same material as Fig. 8. Telophase from one end of an incompletely divided fifth cleavage spindle. A so-called "reconstruction stage," with no sign of fusion of vesicles.

FIG. 10. Same material and stage. The aster at the right end is drawn in position from next section. The distinctness of the chromosomal vesicle at the side is particularly to be noted.



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EXPLANATION OF PLATE II.

FIG. 11. Same material and stage as preceding. Similar conditions shown. Rest of nucleus in next section.

FIG. 12. Same. It is to be noted that the division of the sphere has already taken place for the next mitosis.

FIG. 13. F♀ × C♂, normal material; condition of nucleus before the first cleavage division showing the distinct vesicles. Dotted line marks the area formerly occupied by this nucleus, shrinkage in preparation for ensuing mitosis having already occurred.

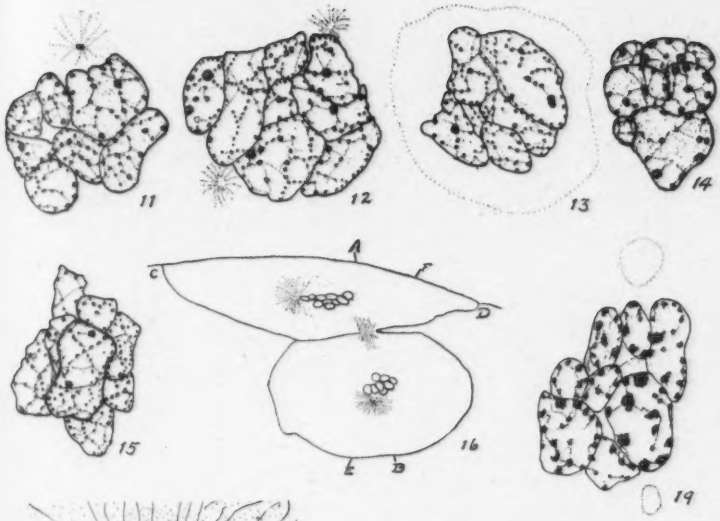
FIG. 14. F♀ × C♂; radiated for three minutes after fertilization. A nucleus from one-cell stage.

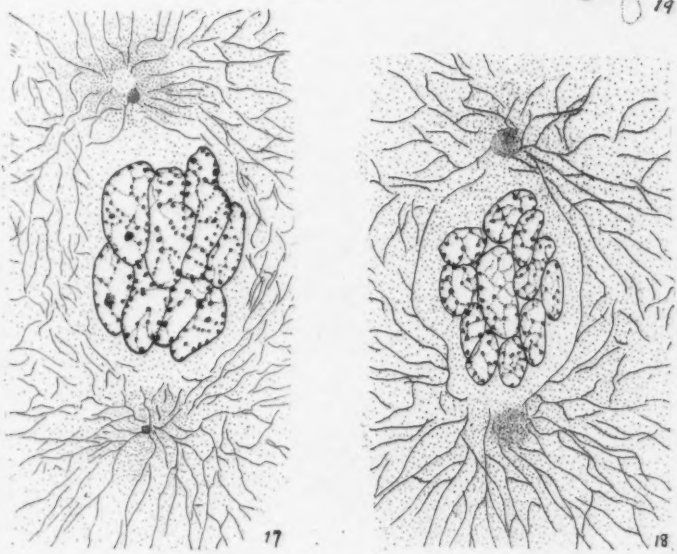
FIG. 15. F♀ × C♂; both radiated for three minutes before fertilization. Typical resting nucleus of second cleavage mitosis.

FIG. 16. F♀ × C♂; drawn with 6 objective and 4 ocular to show change in cell polarity. Blastula stage; upper cell is ectodermal; chromosomal vesicles shown in outline. Original axis was in line *AB*. With the shifting of the position of the spheres in telophase the axis of the upper cell came to line in line *CD* and that of lower in line *EF*.

FIG. 17. F♀ × C♂; eggs radiated for fifteen minutes before fertilization. Beginning of prophase of second division. Vesicles all distinct, showing a very definite polarity. Chromatin granules uniformly small and well distributed. This cannot be interpreted as merely a tangential section showing lobations, for the next section shows quite the same conditions.

FIG. 18. F♀ × F♂; from slide loaned by Miss Pinney as are Figs. 19, 20, 21 and 23. First cleavage similar to Fig. 17.





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EXPLANATION OF PLATE III.

FIG. 19. Pure *Fundulus* material. First step toward chromosome formation is shown in the condensation of chromatin granules on vesicle walls leaving the centers of the vesicles relatively empty.

FIG. 20. Pure *Fundulus* material. Further condensation and beginning of new chromosome as a row of granules, chromomeres, extending out into the center of the vesicle. Drawn as nearly as possible at one level.

FIG. 21. All chromatin granules now formed into the new chromosome anlage. But the vesicular area can yet be distinguished. Only one level drawn.

FIG. 22. F♀ × F♂; radiated three minutes after fertilization. Prophase of third cleavage, showing distinctly old vesicular walls, and also new chromosomes already formed within. Certain of the vesicles show walls especially clearly. Only one level drawn.

FIG. 23. From slide belonging to Miss Pinney. Third cleavage. The only case observed showing the splitting of the chromosomes. The condition of spindle and asters suggest that this splitting may be precocious.

FIG. 24. F♀ × F♂; radiated three minutes after fertilization. Sixth cleavage prophase, oblique section. Traces only of the old vesicular walls are left while the astral rays are making their way into the chromosomes which are more condensed.

FIG. 25. F♀ × F♂; normal. Fourth cleavage, late prophase. As usual, only part of chromosomes are shown. Spindle formation completed and chromosomes are condensing and forming the equatorial plate.

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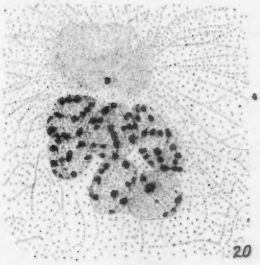

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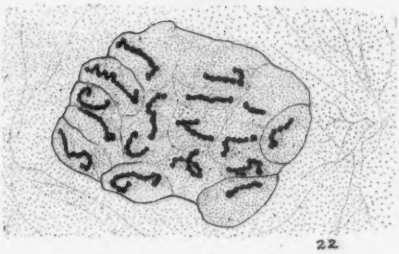

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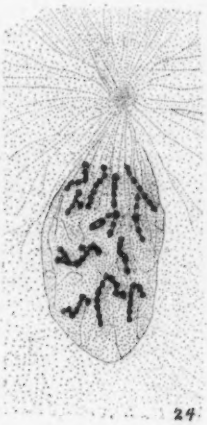

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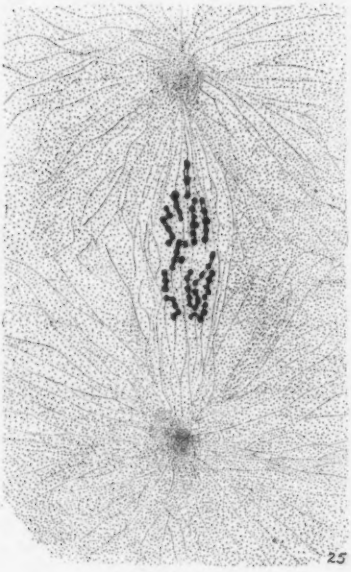

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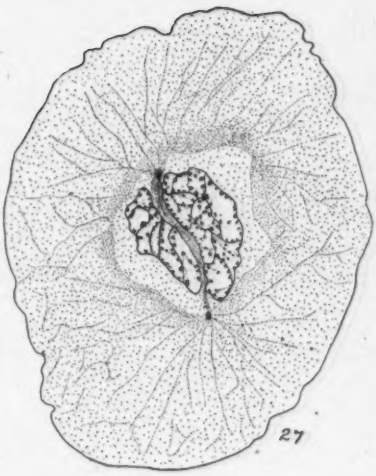
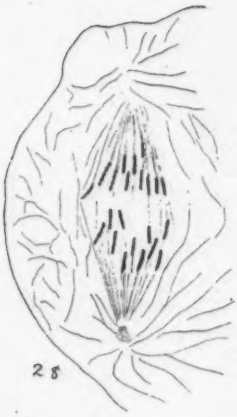
EXPLANATION OF PLATE IV.

FIGS. 26 to 29 are from a grastrulation stage of F♀ × C♂; sperm radiated fifteen minutes before fertilization, by which the sperm chromatin was killed so that it took no further part in development.

FIG. 26 shows the central spindle forming across one side of the nucleus.

FIG. 27 is a later stage showing the central spindle half dividing the nucleus. Not female and male parts, for the sperm's contribution of chromatin was killed as shown in Fig. 29, an anaphase in which all chromosomes are of the *Fundulus* type, and in Fig. 30, which is a cross-section of such a nucleus showing the groove through which the central spindle passes.





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